STRUCTURAL STUDIES OF BOVINE CYTOCHROME OXIDASE SUBUNIT II MESSENGER RNA AND THE CHARACTERIZATION OF ITS INTERACTION WITH THE 28S SUBUNIT OF BOVINE MITOCHONDRIAL RIBOSOMES

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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I dedicate this research to my Lord and Savior, Jesus Christ, to my wife, Janet Marie (Tobin) Courtney and to my sons, Ryan Clark, Sean Michael and Quinn Stephen Courtney.

ACKNOWLEDGMENTS

The people who should be recognized for their support, academic, technical, and emotional would fill most of this and several other sections. Drs. Denslow, Laipis, Lewin, and Nick were of utmost help and encouragement in the pursuit of realizable research as my supervisory committee. Dr. Thomas W. O'Brien, my committee chair, receives my warmest thanks for his endless patience and encouragement, his unusual wit, and his boundless excitement and imagination. I am a different and a better man for the experience and I hope he feels the same. The members of the O'Brien lab, John Anders, Mike Bryant, Wes Faunce, Scott Fiesler, Bob Heck, and Jiguo Liu, have been more than mere co-workers, they have been friends in the truest sense of the word. That friendship has been tested many times, but the mettle of it was never more evident than, when shoulder to shoulder, shivering and aching in the cold room, we were visited with "the way I would do it IF I were doing it." Of these labmates Wes and Mike must be singled out for their meritorious counseling in the face of imminent doom and disaster.

I would like to thank my parents, Bernard G. and Wilma A. Courtney, for instilling in me the desire to always be a

little better and the confidence to go on regardless of the outcome.

Thanks also go to my wife's family for their care, patience, and daughter (and for keeping the cars rolling).

The impact of the joyful, unconditional love of three boys for their father need not be explained, only appreciated and I do. In no manner could I have done this without the quiet strength, support, understanding, endurance and occasional prod of my best friend and wife, Janet.

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ABBREVIATIONS

A 8/6.....messenger ribonucleic acid for subunits 8 and 6 of adenosine 5'-triphosphatase

Ac.....acetate

AMV(-RT)..avian myoblast virus (reverse transcriptase)

ATA.....Aurin tricarboxylic Acid

ATP.....adenosine 5'-triphosphate

ATPase....adenosine 5'-triphosphatase

BSA.....bovine serum albumin

Ci.....Curie

COII.....messenger ribonucleic acid for subunit II of cytochrome oxidase

CPM.....Counts per minute

CPU.....Central processing unit

CTP.....cytosine 5'-triphosphate

CytB.....cytochrome B

dATP.....deoxyadenosine 5'-triphosphate

dCTP.....deoxycytosine 5'-triphosphate

dGTP.....deoxyguanosine 5'-triphosphate

DMS.....dimethyl sulfate

DNA.....deoxyribonucleic acid

DEPC.....diethylpyrocarbonate

ds.....double stranded

dTTP.....deoxythymidine 5'-triphosphate

E. coli...Escherichia coli

eIF.....eukaryotic initiation factor

fMet.....formylated methionine

Fmet-tRNAifMet..methionine initiator transfer ribonucleic acid charged with formylated methionine

FPLC.....fast performance liquid chromatography

GCG.....Genetics Computer Group

GDP.....guanosine 5'-diphosphate

GTP.....guanosine 5'-triphosphate

ICBR.....Interdisciplinary Center for Biotechnology

IF.....initiation factor

kb.....kilobase

kd.....kilodalton

Kd.....dissociation constant

M.....molar

m.....meter

Met.....methionine

Met-tRNAiMet..methionine initiator transfer ribonucleic acid charged with methionine

mg.....milligram

mM.....millimolar

mmol.....millimole

mRNA.....messenger ribonucleic acid

mt.....mitochondria or mitochrondrial

μCi.....microCurie

μl.....microliter

μm.....micron or micrometer

μM.....micromolar

μmol.....micromole

ND.....nicotinamide adenine dinucleotide (reduced)
dehydrogenase

Nm.....nanomolar

nmol.....nanomole

oligo.....oligonucleotide

PCR.....Polymerase Chain Reaction

pM.....picomolar

pmole(s)..picomole(s)

poly(C)...polycytidylic acid

poly(U)...polyuridylic acid

rib(s)....ribosome(s)

RNA.....ribonucleic acid

Rnase.....ribonuclease

RNasin....ribonucleasin, a trademark product of Promega

rRNA.....ribosomal ribonucleic acid

S.....Svedberg

S-D.....Shine-Dalgarno

SDS.....sodium dodecylsulfate

s.....single stranded

tRNA.....transfer ribonucleic acid

tRNAi.....initiator transfer ribonucleic acid

tRNAiMet..methionine initiator transfer ribonucleic acid

tRNAifMet..formylated methionine initiator transfer ribonucleic acid

U.....unit

UTP.....uracil 5'-triphosphate

28S.....mitochondrial ribosomal small subunit

^{32}P.....radioisotope of phosphorous

39S.....mitochondrial ribosomal large subunit

55S.....mitochondrial ribosome monosome

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for Degree of Doctor of Philosophy

STRUCTURAL STUDIES OF BOVINE CYTOCHROME OXIDASE SUBUNIT II MESSENGER RNA AND THE CHARACTERIZATION OF ITS INTERACTION WITH THE 28S SUBUNIT OF BOVINE MITOCHONDRIAL RIBOSOMES

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The translation system of the mitochondrion has many unusual characteristics when compared with other translation systems. Among them are the unique properties of the mRNAS, which lack the 5' cap found in eukaryotic messages and the Shine-Dalgarno sequence of the prokaryotic system. The mitochondrial (mt) ribosome, yet another unique element to this system, must recognize, bind, and translate these atypical mRNAs into proteins. The bovine system was chosen as the model for this research because it is the source of the majority of information on mt ribosomes, and because of the availability of bovine liver for the preparation of mt ribosomes and DNA to be cloned into transcription vectors for the production of mRNAs. The mRNA for COII was chosen as a model study because of its simple monocistronic nature and its bovine mitochondrial origin.

The small (28S) subunit of mitochondrial ribosomes binds the mRNAs tested with unit stoichiometry, one message per 28S subunit and one 28S subunit per message and with a dissociation constant of approximately $5x10^{-8}M$.

The secondary structure of this message was initially predicted using the computer algorithm and subsequently probed with dimethyl sulfate and ribonucleases A, T_1, and V_1 to refine the computer generated model. The use of the same RNA cleavers and modifiers in the presence of 28S subunits provided "footprints" on the COII mRNA of their interaction site(s). A region of multiple cleavage reductions (7-13 nuclease susceptible bases) was found from base 288-314 with additional T_1 reductions observed elsewhere in the molecule (guanosines 23, 24, 92, 195, 205, 209, 356, 410, 440, 453, and 457), possibly effects peripheral to the actual binding. Ten sites of enhanced sensitivity were also seen for RNase A throughout the molecule, but particularly between 210 and 365 in the presence of ribosomes. Some of these may indicate areas in the RNA that have been made more available for cleavage as a result of the interactions between the 28S subunit and the COII mRNA.

Thus, a high affinity interaction between COII mRNA and the small ribosomal subunit has been characterized. The COII mRNA displays a potential 30+ nucleotide binding site consistent with the previously described binding site on the 28S subunit.

BACKGROUND

The process by which proteins are synthesized is a three tiered pathway that was predicted by James Watson, as simply "DNA makes RNA makes protein." This along with "the assertion that information passed to protein cannot return to its former state" (i.e., proteins do not carry information for their own biosynthesis), (T. Hunt et al., 1983), form the bases for the Central Dogma. While this wonderfully simplifies the protein biosynthetic process, the individual steps in the process are rife with complexities which are inconsistent with the term dogma. The diversity between the prokaryotic and eukaryotic systems in the components and the methods used to complete the protein synthetic process are differences that have and will continue to be at the center of much investigative effort.

The first step in the process is the transcription of DNA into RNA, ribosomal, transfer, and messenger RNAs. Ribosomal RNAs (rRNAs) are the functional skeleton around which ribosomal proteins assemble to form the two subunit translation organelle, the ribosome. Three (or four for eukaryotes) rRNAs are used in this ribonucleoprotein complex and, in the case of prokaryotic ribosomes, these rRNA molecules participate more directly in the translation

process. The single strand nature of these molecules and their inherent base pairing potential allow these, as well as any RNA molecule, to form secondary and tertiary base pairing interactions (Gutell et al., 1985).

In rRNAs the secondary structures are extensive and fairly well conserved, which provides for a stable long-lived molecule upon which to build a ribosome. Transfer RNAs (tRNAs) are small (70-130 bases) highly structured (secondary and tertiary) RNAs that serve a dual function in the translation process. They decode the information contained in messenger RNAs (mRNAs) with a three base anticodon sequence which specifies a particular amino acid and complements a codon sequence on the mRNA. They also deliver that particular amino acid to the ribosome for addition in polypeptide chain elongation. These RNAs have been used extensively as models for the development of computer algorithms for predicting secondary structure in RNA molecules. The mRNAs are carriers of the genetically encoded information for the synthesis of proteins from its origin in the genome (DNA) to its site of translation, the ribosome.

Messenger RNAs may be quite complex in their primary and secondary structures. The primary structure can be complicated by having its coding regions, exons, separated by intervening non-coding regions, introns, by having a 5' cap, or by having 5' and 3' untranslated regions. The exons must then be spliced together and the introns removed prior to

translation of the message into protein. These RNAs are also capable of forming secondary and tertiary structures. In several cases, the secondary structure of mRNAs has been shown to be important in the regulation of their translation, either providing a binding site for a regulating element or preventing or enhancing the ability of the ribosome to bind, move on the mRNA, or initiate protein synthesis (Kozak, 1990; Haile et al., 1989).

The final step, translation, can be subdivided into three phases: initiation, elongation, and termination. While the product is the same regardless of which system is being observed, prokaryotic or eukaryotic, and the central characters maintain the same roles, the cast of supporting players can diverge considerably. In prokaryotic systems initiation requires mRNA, IF1, IF2, IF3, fMet-$tRNA_i$, GTP, and both ribosomal subunits, whereas eukaryotic systems usually operate with 5' Capped mRNA, eIF1, eIF2, eIF3, eIF4F, eIF5, Met-tRNAiMet, GTP, ATP, and both ribosomal subunits (see Figure 1, Darnell et al., 1990). This situation also reveals that initiation mechanisms differ between prokaryotes and eukaryotes. Each system assembles its components and factors so that the end result is an initiation complex comprised of a ribosome with a charged $tRNA_i$ at the peptidyl-tRNA site and an mRNA in position for decoding. In all stages of translation, the ribosome remains the central component of all biosynthetic activities in both systems, prokaryotic and

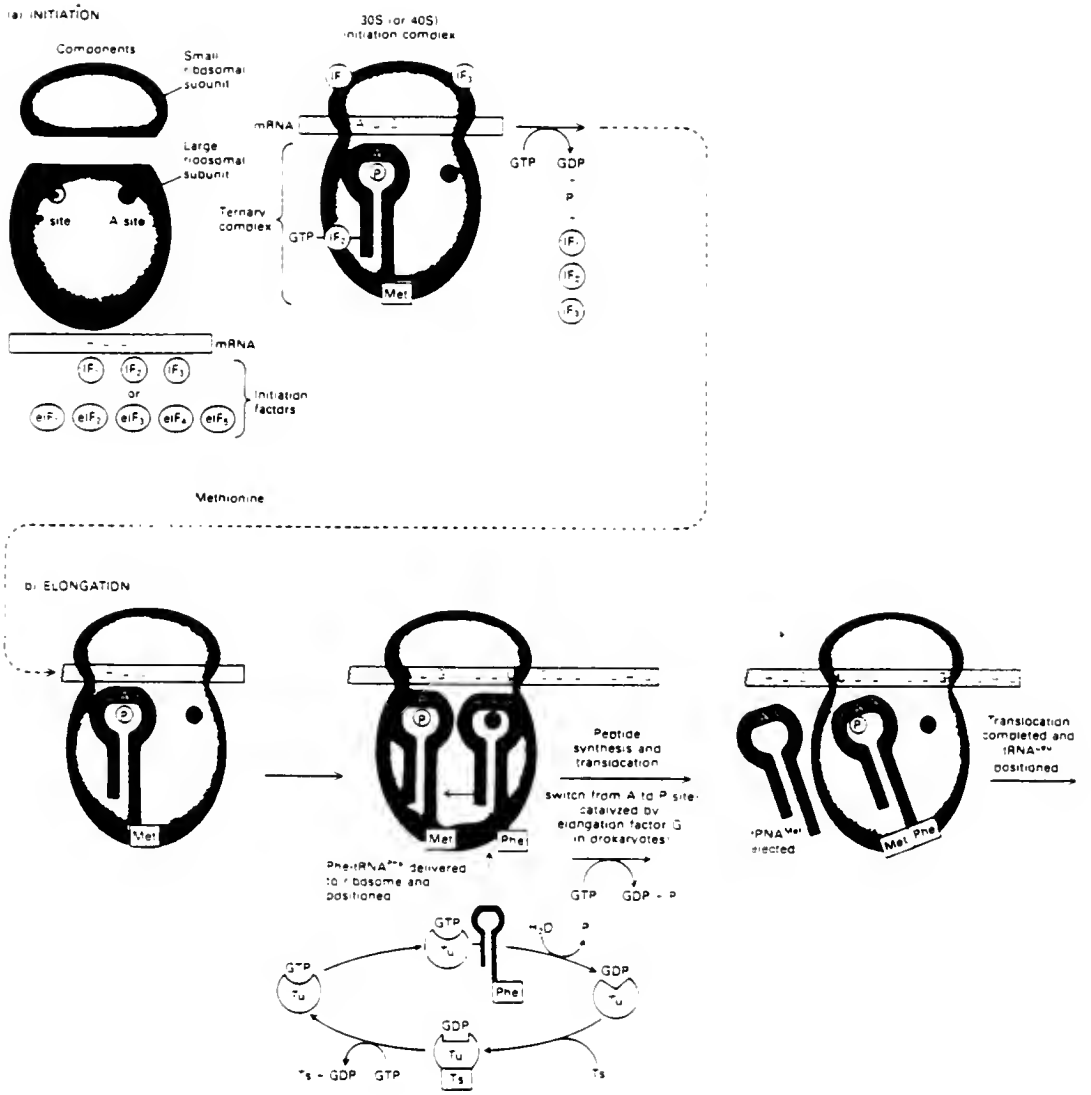

Figure 1. The Three Stages of Translation (Darnell et al., 1990). Translation is divided into three stages, initiation, elongation, and termination. Each stage has its own particular requirements for substrates and factors that differ depending on the type of ribosome.

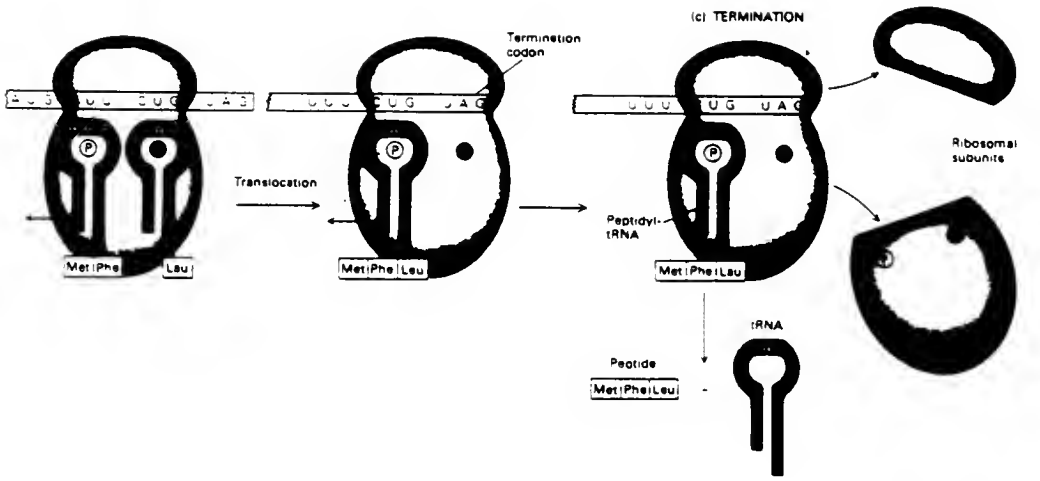

Figure 1 (cont.)

eukaryotic. Both systems possess ribosomes, albeit with unique characteristics.

A third and physically unique kind of ribosome was reported in 1967, the 55S mitochondrial ribosome (O'Brien and Kalf, 1967; O'Brien, 1971). For years skeptics remained unconvinced of this finding, considering the 55S "mini ribosome" particle to be a bacterial contaminant or residual cytoplasmic large subunit contaminant. As the data substantiating its existence grew with time, the 55S ribosome eventually won acceptance as part of a semi-autonomous mitochondrial genetic system and represents a unique class of ribosome. Subsequent investigations showed the factors participating in the bacterial protein synthetic process are not always interchangeable with those normally used by the 55S ribosome (Denslow and O'Brien, 1979) indicating that the 55S ribosomes had properties different from bacterial ribosomes. Since that time, the sequences of many of the mammalian mitochondrial genomes have been published (Anderson et al., 1981, 1982; Bibb et al., 1981). These DNAs encode rRNAs, tRNAs, and ,in the case of mammals, 13 open reading frames, two of which are bicistronic (Chomyn et al., 1985). Some mitochondria specific factors have been isolated (Liao and Spremulli, 1990a), and even a few of the ribosomal protein genes have been sequenced (Liu and O'Brien, personal communication). Twenty-five years later, the inquiry into the 55S ribosome and the mitochondrial translation system has left

many of these unique aspects an enigma, as 80S and 70S ribosomes continue as the preferred models in the study of translational systems.

This notwithstanding, the mitochondrial translation system demands elucidation by virtue of its various unique properties (see Table 1). The mitochondrial ribosome is characterized by a low sedimentation rate of 55S, imparted by its relatively high protein content (85 proteins total) and a larger mean size of ribosomal proteins by comparison with prokaryotic ribosomes (53 proteins total)(Matthews et al., 1982). 55S ribosomes are assembled around a 12S rRNA and a 16S rRNA (corresponding to the 16S rRNA and 23S rRNA for prokaryotic ribosomes). Current speculation suggests that the "extra" proteins found in 55S ribosomes may fill the voids created by the shorter mitochondrial rRNAs (O'Brien et al., 1980).

Mitochondrial mRNAs are remarkably different from those of the other translation systems. Mitochondrial mRNAs, with the exception of the message for nicotinamide adenine dinucleotide (reduced) dehydrogenase subunit 6 (ND6), are transcribed as a large (16+ kilobase), continuous RNA representing the entire mitochondrial genome. ND6 and a few of the tRNAs are transcribed in a similar manner from the light strand. These transcripts have the unusual properties of being intronless and having their open reading frames punctuated by tRNAs. The current theory is that the tRNAs

Table 1

Unique Properties of Mammalian Mitochondrial Translational Components (O'Brien, 1971, O'Brien et al., 1980, Gaines and Attardi, 1984, and Denslow et al., 1991).

1. Sedimentation rate of 55S
2. High Protein Content
3. Low rRNA content
4. No 5' methyl cap or leader sequences
5. No Shine-Dalgarno Sequence
6. Unique (and possibly resident) factor requirements
7. Unique genetic code and tRNAs
8. Mitochondrial ribosomes are products of two genomes
9. All products of mitochondrial translation are residents of the inner mitochondrial membrane
10. Mitochondrial ribosomes bind GTP directly
11. Mitochondrial mRNAs contain no introns
12. Mitochondrial mRNAs have neither 5' nor 3' untranslated regions
13. Mitochondrial mRNAs are transcribed as a large 16kb RNA that is processed into rRNAs, tRNAs, and mRNAs.

provide recognition sites for the nearly immediate processing of the large transcript RNAs into their constituents of 11 mRNAs, 22 tRNAs, two rRNAs, and a 7S RNA of unknown function (Ojala et al., 1981). The mRNAs have neither 5' methyl-G caps nor S-D leader sequences (Shine and Dalgarno, 1974), nor does the 12S rRNA possess an anti-S-D sequence. In fact, mitochondrial mRNAs possess little or no 5' leader sequences (see Figure 2) and no 3' untranslated sequence (Montoya et al., 1981). Since 5' caps and leader sequences are both absent, mitochondrial translational initiation must take place through a novel and yet to be defined mechanism. Speculation has suggested the presence of an internal S-D like sequence (Saccone et al., 1985), while others speculate that the 5' ends of the mRNAs are important (Denslow et al., 1989). In any event the function of facilitating the localization of the initiation codon in the decoding site, provided by the S-D site on the 5' ends of prokaryotic mRNAs must be the function of a separate and distinctive factor particular to the mitochondrial translation system. Perhaps the mitochondria possess a protein similar to S1, which maintains the message on the small subunit of E. coli until the S-D sequence can function and the initiation complex can form (Calogero et al., 1988). Alternately, the mRNAs may provide for "internal ribosome entry sites" as those found for poliovirus mRNAs in their 5' untranslated terminal repeat. Simply, it may be that the binding affinity is of sufficient strength to allow for

```
                +1
ATPase 8         C AUG CCGCAACUAGACAC

                +1
COI              C AUG UUCAUUAACCGCUG

                  +1
COII               AUG GCAUAUCCCAUACA

               +1
COIII           UA AUG ACAACCAAACUCAU

               +1
ND1             AA AUG UUCAUAAUUAACAU

                  +1
ND2                AUA AACCCAAUUAUCUU

                  +1
ND3                AUA AAUUUAAUACUAGC

                  +1
ND4                AUG UCUAUAGUAUACAU

                  +1
ND5                AUA AACAUAUUCUCCUC

                  +1
ND6                AUG AUACUAUACAUUGU

             +1
CYTB          ACUA AUG ACUAACAUUCGAAA
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Figure 2. 5' Ends of Bovine Mitochondrial mRNAs. Note the extremely short, or non existent, 5' leader sequences (Montoya et al., 1981; Hill et al., 1990). The +1 indicates the first base of the message and start codons are underlined.

the proper positioning of the message on the small subunits, in the vicinity of the decoding site as predicted by experiments on prokaryotic messages with or without S-D sequences (de Smit and Van Duin, 1990). The 55S ribosome may recognize a particular secondary structure in the mRNA near the 5' end, though no experimental evidence for the theory exists. The effect of the 5' end structure, whether 5'-OH, monophosphate or triphosphate, has been found to play no role in the actual binding of the COII mRNA by the 28S subunit (Liao and Spremulli, 1990a). The whole story is likely to be more complicated, since the mitochondrion translates two messages which are bicistronic and out of frame: ATPase 8/6 and ND 4L/4.

One possibility for the mechanism by which the mitochondrial ribosome initiates translation is the presence of a sequence nonspecific RNA binding domain on the mitochondrial ribosome (Denslow et at., 1989). The roughly thirty base long binding domain of this binding site (see Figure 3) was disclosed by oligoribonucleotide binding studies to determine the affinity of polynucleotides of increasing lengths from 3-42 bases. Furthermore, an RNase protection domain of up to eighty bases in length was provided on bound RNA (5' end labelled). Current thinking suggests this domain may aid in positioning the mRNA on the mitochondrial ribosome for the start of translation and may stabilize the mRNA during elongation. To examine the processes of initiation and

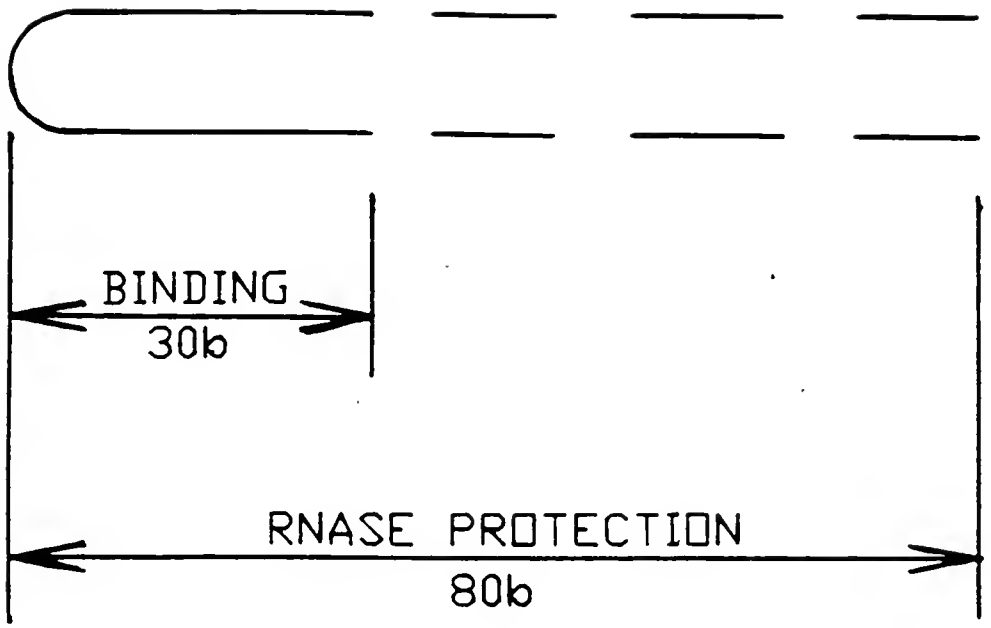


Figure 3. The RNA Landing Pad on Mitochondrial Ribosomes. Messenger RNA is thought to bind to a domain on the small subunit roughly 30 bases in length. An 80 base domain of protection from nuclease activity is provided by the subunit (Denslow et al., 1989).

elongation, a few mitochondrial translation factors have been isolated (Liao and Spremulli, 1990a), although the majority of factors probably remain to be found. The peculiarly large number of ribosomal proteins in the 55S ribosome allows for the possibility of "resident" factors, factors that are in constant association with the ribosome.

In addition to a unique initiation mechanism, the 55S ribosome translates mRNA using a unique genetic code and unique mitochondrially encoded tRNAs. This ribosome utilizes fewer tRNAs (22) than its bacterial counterparts (61). Interestingly, the search for a mitochondrial initiator tRNA has been a difficult one and to this point fruitless. While the mitochondrial genome encodes a methionyl-tRNA, no evidence of its ability to be processed (formylated) into an initiator tRNA has been found. Initiator tRNA obviously exists and the possibility of its import into the mitochondria has been considered. Keeping in mind the known properties of the mitochondrial system, it seems likely that its elongation mechanism may also prove unique.

Another unusual property of mitochondrial ribosomes is their assembly from two different genomes. The proteins are encoded by the nuclear genome, translated on 80S ribosomes, and then imported into the mitochondria (Schieber and O'Brien, 1982) where they are assembled with the rRNAs, transcribed from the mitochondrial genome, to form 55S ribosomes. This mechanism of ribosome biosynthesis raises a number of

questions unique to the mitochondrial translation system: What are the control mechanisms for the maintenance of stoichiometric levels of ribosomal proteins to rRNAs and what is the method of communication between the mitochondrial machinery and that of the cytosol and the nucleus?

Another distinctive property of 55S ribosomes is that they bind GTP directly (Denslow et al., 1991). Figure 1 shows that GTP is involved in a number of steps in the translation mechanism of prokaryotes and eukaryotes, but in no step does the GTP bind to the ribosome directly. Experimentation has shown that GTP binds to the 55S ribosome tightly (Kd = 20 Nm) and exchanges rapidly with GDP, which has an apparently equal affinity for the ribosome. There is, however, no evidence yet for a functional role for GTP or GDP binding, but the binding of GTP directly to the ribosome provides circumstantial evidence for the presence of a resident factor. Understanding the GTP binding function evokes still another path for further consideration.

Although the focal points of study on translation have revolved around the eukaryotic and prokaryotic systems, the many unique properties of the mitochondrial translation system, outlined in Table 1, also demand attention. This is a fundamental work inasmuch as the 55S ribosome and some initiation and elongation factors are the only members of the mitochondrial protein biosynthetic mechanism to have been described. This rationale was the basis for earlier research

on the RNA molecule of the small subunit by W. Faunce, and ongoing work by R. Heck on the RNA interaction site on the 28S subunit, and J. Liu on the function of GTP binding. So one might logically be directed to the "first step," initiation, which has as an early step, the binding of mRNA by the 28S subunit. While the ribosome binds the mRNA, the message is being bound and these are the points of interest of the research to be described here. The work done on the characteristics of mRNAs/ribosome interactions in the prokaryotic and eukaryotic systems may provide clues to the important features to expect in the mitochondrial system. How can this interaction between the 28S subunit and the mRNA be elucidated?

Essential questions to be addressed in this research are as follow: Is the binding of mitochondrial mRNAs in any way specific? Does the 28S subunit bind all mRNAs similarly? Does it bind all polynucleotides equally? What is the binding stoichiometry? Where is(are) the ribosome binding site(s) on mRNA? What are the characteristics of the mRNA where it is bound by the ribosome?

Binding studies can be employed using a number of polynucleotides to determine the extent of specificity, the strength of the interaction, and its stoichiometry. An often used filter binding assay provides a quantitative yet quick and inexpensive mode for determining the strength of interaction and specificity of a number of ligands,to include

mitochondrial mRNAs for cytochrome oxidase subunit 2 (a monocistronic mRNA) and ATP synthase subunits 8 and 6 (a bicistronic mRNA). The binding stoichiometry of 28S subunits on mRNA can be analyzed by centrifugation in sucrose density gradients, the method used in the isolation of 28S subunits preparatively.

Some insights into the sequence/structural properties of the mRNA at the site of interaction with the 28S subunit may be revealed in the specificity studies, comparing the properties of polynucleotides that bind to those that do not. Additionally, the development of computer predictions of the secondary structure of a 5' leaderless model mRNA, COII (Figure 2), and one that has been used in previous mitochondrial initiation studies may provide some clues to the putative site of interaction. It would be naive to accept the results of an RNA folding program carte blanche, especially when the majority of folding programs is based on the structures of tRNAs.

Therefore, the mRNA must be probed in some manner to confirm or refine the computer prediction. The techniques most frequently used to study the tertiary structure of macromolecules are X-ray crystallography and electron microscopy. X-ray crystallography is exceedingly time consuming, expensive and has yet to be successfully applied to RNA structures. Electron microscopy is limited in its ability to disclose structural details in molecules like large RNAs.

The method which most efficiently discloses the secondary structure of an RNA is the modification of the RNA followed by primer extension termination (Moazed and Noller, 1986; Stern et al., 1988). This primer extension technique involves modifying the COII mRNA with either enzymes or chemical agents. The modified mRNA is then purified and annealed to one of several synthetic oligodeoxyribonucleotide primers complementary to regions spaced at intervals of approximately 150 bases. The annealed primers are then extended using reverse transcriptase (Boorstein, 1989; Knapp, 1989). The extension is disrupted at the modified bases, providing a means of disclosing the sites of modification. Finally, the primer extension products are analyzed on a denaturing gel to identify the modified bases.

The final question is answered using the same primer extension technique, but in the presence of 28S subunits. This technique gives a "footprint" on the mRNA by first allowing the 28S subunit to bind the message, followed by chemical modification or enzymatic cleavage. Then, as before, primers are annealed and extended with reverse transcriptase. The interruptions to extension are again disclosed by denaturing gels and the difference in modifications/cleavage in the presence and absence of 28S subunits is analyzed to disclose the "footprint" (sites of blocked modification/cleavage).

In this study, mitochondrial mRNA was analyzed using a chemical modifier, dimethyl sulfate (DMS) (Donis-Keller et al., 1977), which reacts primarily with unpaired adenines and cytosines, and either paired or unpaired guanines (see Figure 4)(guanines are disclosed as modified by chemically cleaving the purine ring, a technique not used in this work). In addition, the COII mRNA was probed using RNase A (which cleaves 3' to pyrimidines in single-stranded RNA), RNase T_1 (which cleaves 3' to unpaired guanines), and RNase V_1 (which cleaves primarily double stranded RNA)(Gehrke et al., 1983). Chemical as well as enzymatic modifiers were chosen to allow for an additional level of analysis. First, the secondary structure of the mRNA could be investigated by the use of single-strand specific modifiers (RNases A and T_1, and DMS) or a double strand specific modifier (RNase V_1). Secondly, the protection from modification/cleavage in the presence of 28S subunits provides a "footprint" of the binding site. Finally, the disposition of the mRNA within in the binding site of the 28S subunit and its own secondary/tertiary structure can be studied. The relatively bulky enzymatic modifiers (the RNases) may only be effective on ribonucleotides exposed on the surface or not in association with the small subunit. The smaller chemical, however, should penetrate through the secondary/tertiary structure of the 28S subunit to methylate most pyrimidines not involved in Watson-Crick base pairing or tertiary interactions.

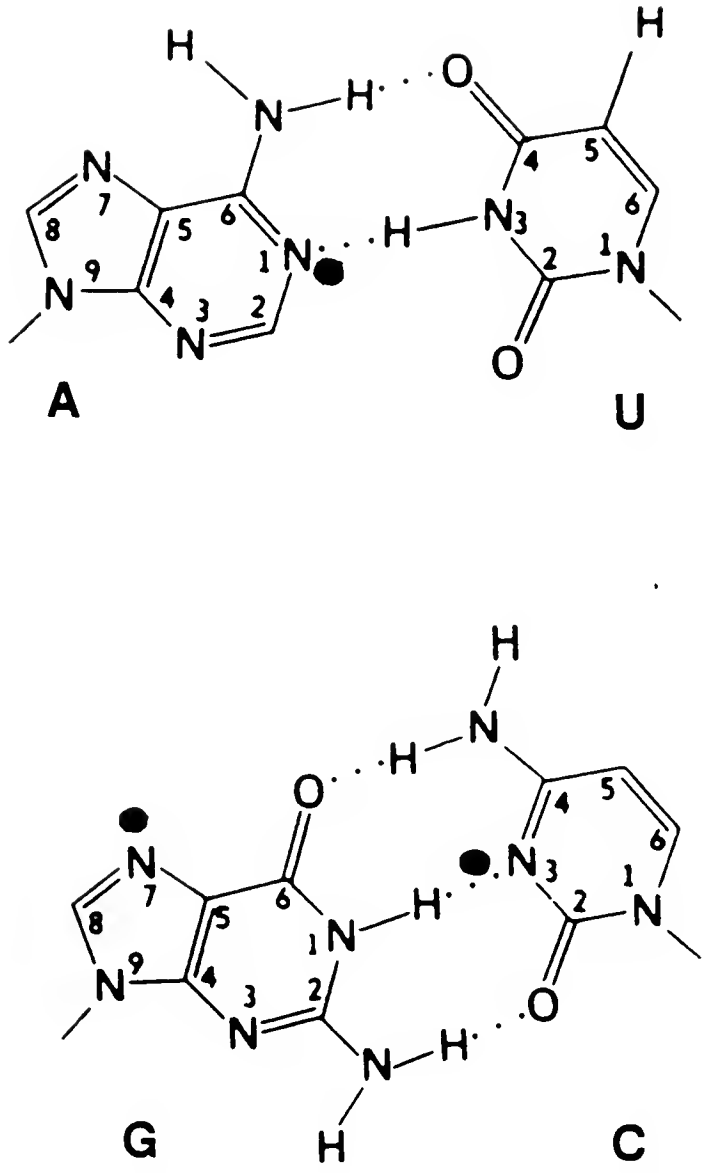


Figure 4. Site of Chemical Modifications on Ribonucleotides. Dots represent the sites of modification by DMS. All modifications are blocked by Watson-Crick base-pairing except the modification of guanine.

With these tools in hand, some of the questions pertaining to the initiation of translation within the mitochondrion can be approached. Does the binding of bovine mitochodrial mRNAs require the presence of additional factors? Is the binding strength significant for physiological function? What is the stoichiometry of binding? What specific attributes are necessary on messages for binding by 28S subunits? What is the nature of the mRNA at its site(s) of interaction with the small subunit? These are the questions to be answered in this research. The results and the techniques used can be successfully employed in future studies concerned with initiation of translation in the bovine mitochondrial system.

MATERIALS AND METHODS

Preparation of Bovine Mitochondria

Bovine mitochondria were isolated from fresh liver (less than 30 minutes post mortem and provided for the most part by the University of Florida Meat Lab) which was sliced thinly (approximately 1.0 cm thickness) on location and submerged in isolation medium (0.34 M sucrose, 1 mM EDTA, and 5 mM Tris-HCl, pH 7.5 at 0-4°C), as described previously (Matthews et al., 1982). The tissue and subsequent mitochondria were maintained in this buffer at this temperature throughout the preparation. The sliced liver was ground coarsely in a commercial meat grinder and then homogenized in a flow-through homogenizer (Tekmar).

Mitochondria were resuspended to a concentration of 20 mg protein/ml in isolation medium, treated with 100 μg/ml digitonin, final concentration, and stirred for 15 minutes. The digitonin-treated mitochondria (mitoplasts: mitochondria lacking the outer membrane) were then diluted 5-fold in isolation medium and collected by centrifugation at 11,000 x g for 10 minutes. Mitoplasts were washed four additional times in isolation medium and stored at -70°C in freeze buffer

(40mM KCl, 15mM $MgCl_2$, 10mM Tris-HCl, 5mM 2-mercaptoethanol, 0.05mM EDTA, 0.05mM Spermine, 0.05mM Spermidine, pH 7.5).

Preparation of Native 28S Subunits and 55S Monosomes

Mitochondrial ribosomal native 28S subunits were prepared according to the method described by Matthews et al., 1982. Mitochondrial ribosomes were isolated from the mitochondrial lysate and separated in a 10-30% sucrose density gradient in 20mM $MgCl_2$, 100mM KCl, 5mM 2-mercaptoethanol, and 10mM triethanolamine, pH 7.5. The gradients were monitored (UV) and fractions were collected (Figure 5). The fractions containing 55S monosomes, and 28S and 39S subunits were pooled separately and the particles were concentrated by ultracentrifugation, 45,000 rpm for 18 hours in a Beckman Ti55.2 rotor. The subunit pellets were resuspended subunit freeze buffer (25mM KCl, 2.5mM $MgCl_2$, 10mM TEA and 5mM 2-mercaptoethanol, pH 7.5) and monosomes were stored in monosome freeze buffer (25mM KCl, 5mM $MgCl_2$, 10mM TEA and 5mM 2-mercaptoethanol, pH 7.5) and stored at -70°C until needed.

Preparation of Derived 28S Subunits

The 55S monosomes were dissociated on 10-30% sucrose density gradients in 5mM $MgCl_2$, 300mM KCl, 5mM 2-

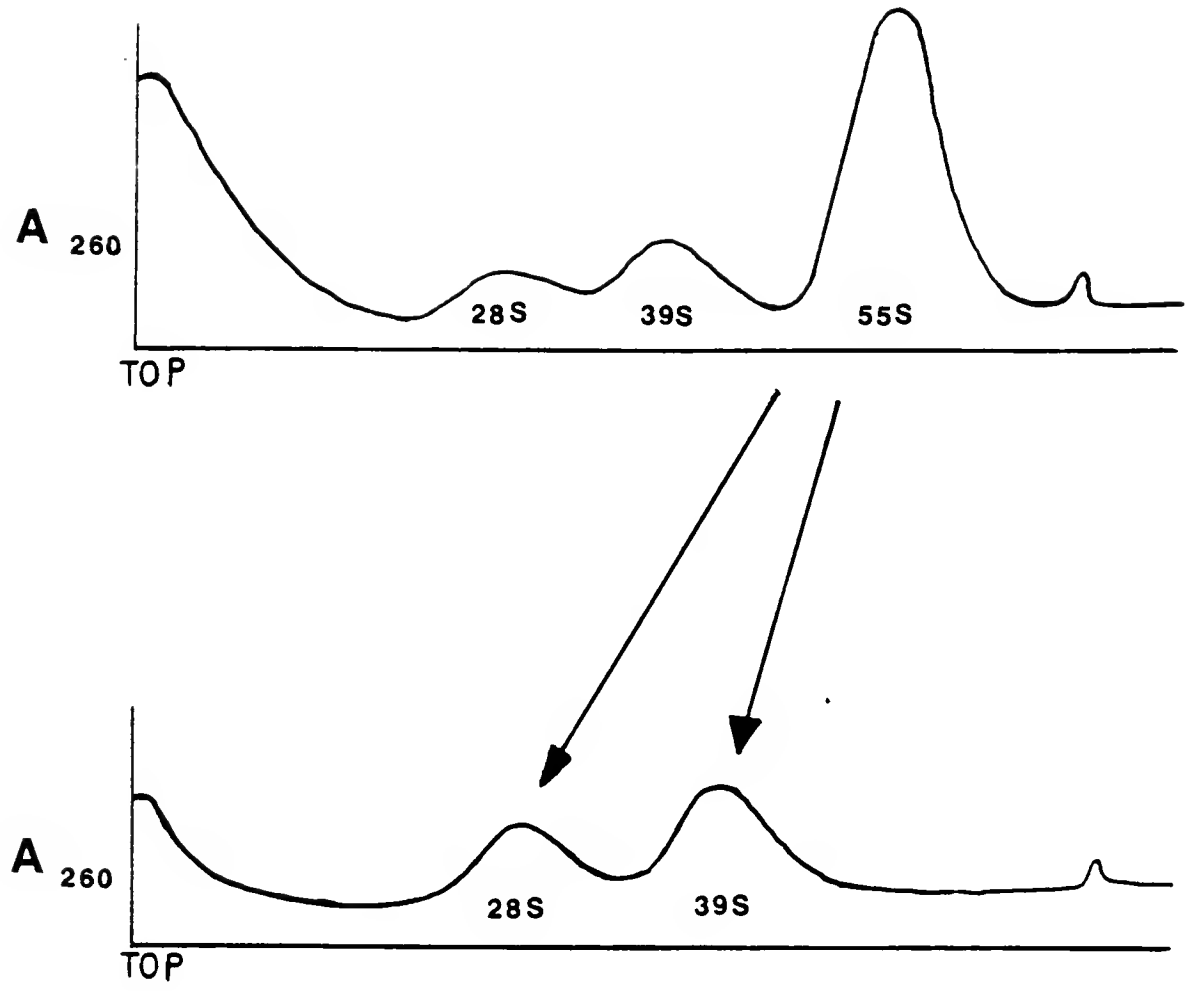


Figure 5. Absorbance Profiles (260nm) of Ribosomes in Sucrose Density Gradients. The upper gradient renders "native" subunits and the lower gradient "derived" subunits. Note the separation between each subunit and the monosome.

mercaptoethanol, and 10mM tris-HCl, pH 7.5. The derived 28S subunit fractions were then pooled and the particles were concentrated by high speed centrifugation, 45,000 rpm for 18 hours in a Beckman Ti55.2 rotor, resuspended in subunit freeze buffer and stored at -70°C until used.

Preparation of Mitochondrial DNA

Mitochondria (4 grams protein) were concentrated by centrifugation at 11,000 x g for 10 minutes and resuspended in STE buffer (100mM NaCl, 50mM Tris-Cl, pH 8.0, and 10mM EDTA). These mitochondria were then lysed by the addition of SDS to 1% (w/v) final concentration. The mitochondrial lysate was then subjected to cesium chloride (CsCl) gradient centrifugation at 50,000 rpm in a Beckman TY65 rotor for 24 hours in the presence of ethidium bromide (EtBr). Two UV luminescent bands are discernible at the completion of centrifugation. The topmost and thinner band (containing mitochondrial DNA) was removed from the gradient and the DNA was ethanol precipitated by adding 2.5 volumes of 95% ethanol and kept at -70°C for at least one hour. The precipitate was pelleted, washed four times with 70% EtOH, dried, and resuspended in TE buffer (10mM Tris-Cl, pH 8.0, and 0.1mM EDTA) to a concentration of 7-10μg/ml, with 30μg total yield, and stored at -20°C.

Cloning Mitochondrial Genes

The mitochondrial DNA was digested by HindIII restriction endonuclease to produce three fragments: 10.2, 4.4, and 1.7kb. These fragments were cloned individually into a pUC 19 vector linearized by HindIII restriction endonuclease for amplification (as described Current Protocols in Molecular Biology), storage, and mutagenesis. Clone 38 (Figure 6) contained the 4.4kb fragment, which includes the gene for ATPase 8/6. This clone was used to prepare the transcription vector for subsequent mRNA production. In order to make an mRNA that would most closely approximate the known mRNA sequence, oligonucleotide-directed mutagenesis was utilized (Kramer et al., 1984). Oligonucleotides (oligos)(Table 2) were made by the DNA Core Facility, ICBR, to anneal to the 5' and 3' ends of ATPase 8/6 gene in opposing orientation (Figure 7) to utilize the polymerase chain reaction. These oligonucleotides were made up of 18 bases on their 3' ends which complemented the 5' or 3' end of the gene exactly. Each contained recognition sequences for a number of restriction endonucleases (see figure 7) for cloning and, later, for transcription purposes. The products of the PCR were incubated with BamHI and EcoRI restriction endonucleases and isolated by gel electrophoresis on a 1.5% low melting temperature agarose. After EtBr treatment (0.1μg/ml) the UV luminescent band corresponding to approximately 900 base pairs

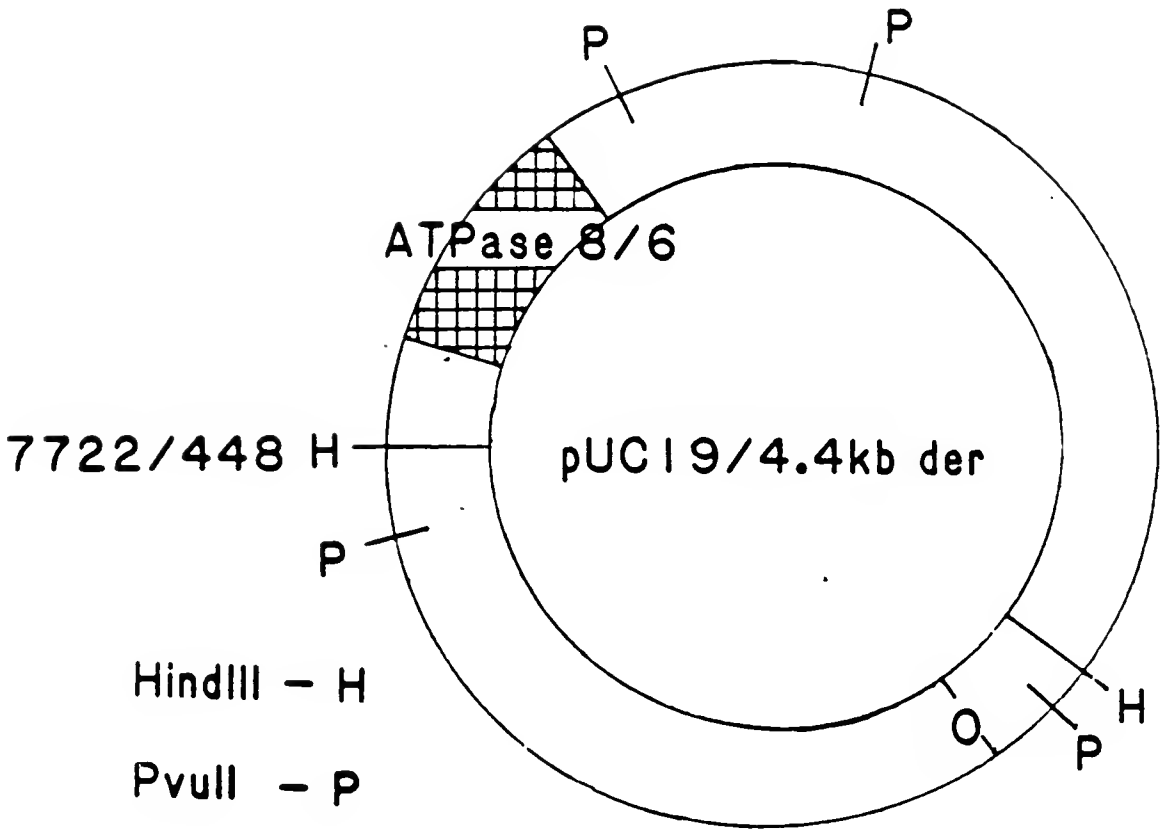


Figure 6. Map of Clone 38 of ATPase 8/6 for Mutagenesis. pUC19 vector with a 4.4kb HindIII fragment from the bovine mitochondrial circular DNA (>16kb).

Table 2

Oligonucleotides and their Uses.

Oligonucleotide	Use
TO 27	Mutagenesis of ATPase 8/6
TO 28	Mutagenesis of ATPase 8/6
TO 136	Primer Extension Ω of COII mRNA
TO 135	Primer Extension α of COII mRNA
TO 134	Primer Extension β of COII mRNA
TO 133	Primer Extension γ of COII mRNA
TO 132	Primer Extension δ of COII mRNA
TO 139	Binding Substrate
TO 105	Binding Substrate

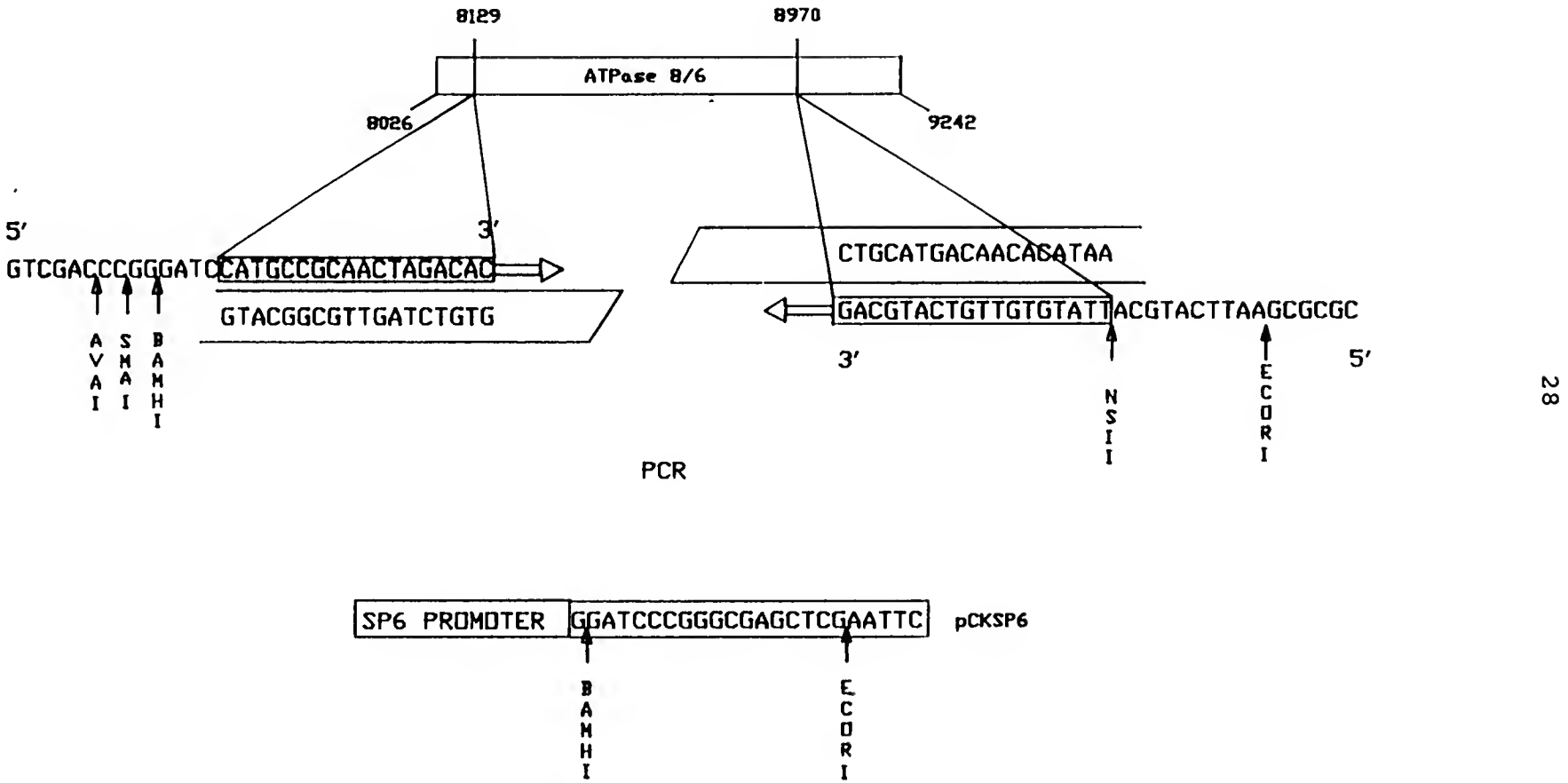


Figure 7. Oligonucleotide-directed, Site-specific Mutagenesis in Conjunction with Polymerase Chain Reaction. A) Oligonucleotides base pair exactly with 18 bases on opposite strands at the 5' and 3' ends of the molecule. They contain the recognition sequences for several restriction endonucleases B) Annealing primers to denatured and linearized template. C) Extension and amplification by PCR using TAQ polymerase. D) Restriction digest with BamHI and EcoRI and ligation into a prepared (BamHI and EcoRI digested) pSPK6 transcription vector.

was removed from the gel. The transcription vector pCKSP6 (a gift of Dr. C.W. Wu, State Univ. of New York at Stony Brook) (1μg), linearized by the same two restriction endonucleases, was purified from its polylinker fragment by EtOH precipitation in 2.5M NH_4OAc at -70°C and two subsequent washes with 70% EtOH. This vector was then resuspended and added to the gel piece containing the digested PCR product. The solution was brought to a final volume of 50μl with ligase buffer (40mM Tris-HCl, 10mM $MgCl_2$, and 1mM DTT) and warmed to 65°C until the agarose melted and then cooled to 37°C. T4 DNA Ligase (10U) was then added and the mixture was incubated for three hours at 37°C. This product was transfected into E. coli (TG-1 and JM109), amplified, and stored as 50% glycerol stocks at -70°C for further use.

Engineering an ATPase 8/6 Transcription Vector

The pCKSP6 vector containing the ATPase 8/6 gene required further manipulation before it could be used in the prescribed manner to generate an mRNA transcript. This was accomplished by linearizing the circular DNA with BamHI restriction endonuclease and removing the four base 5' overhanging ends with S1 nuclease, a 5'-3' single strand specific exonuclease (Figure 8). The clone was then re-circularized by T4 DNA Ligase, transfected into E. coli strain TG-1, amplified, and selected for by loss of the BamHI restriction endonuclease

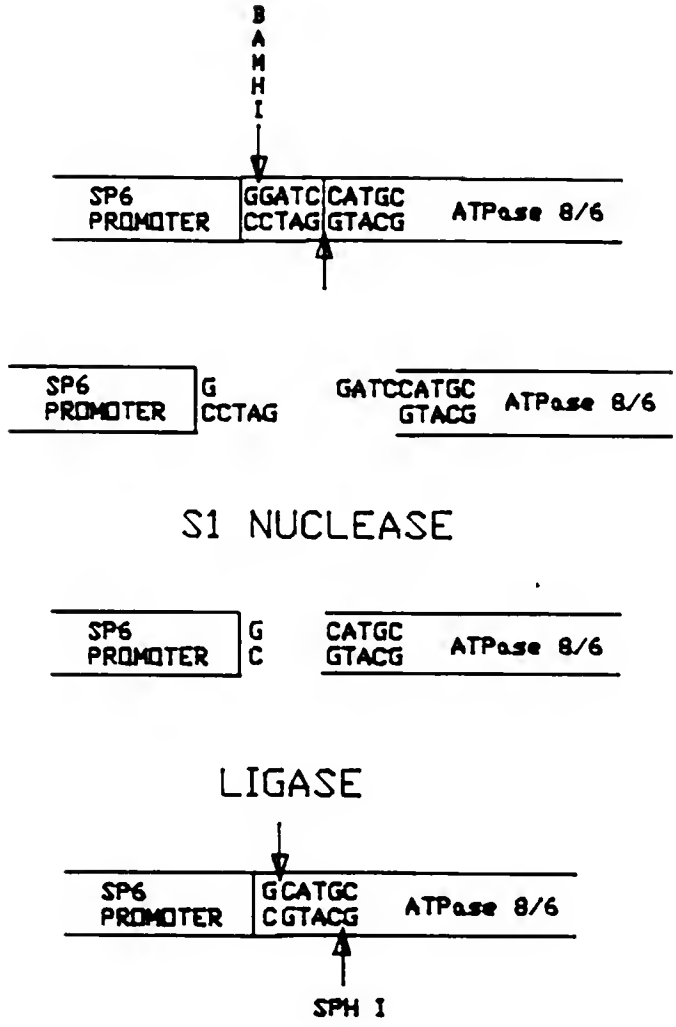


Figure 8. Engineering the ATPase 8/6 Clone. A) Linearizing with BamHI. B) Excision of 5' end overhangs and ligation of the blunt ends (re-circularizing the vector). C) pJC213 with new SphI restriction site at the 5' end of the gene.

site and the addition of an SphI restriction endonuclease site. The clone, pJC213, was later sequenced using Thermus aquaticus (TAQ) polymerase and dideoxynucleotide triphosphates (as described in PCR Protocols, Innis et al., 1990) for verification. Clones that were positive for these two traits were then sequenced with TAQ DNA polymerase for confirmation. pJC213 was the clone selected for further use in RNA synthesis.

Preparation of Transcription Vectors

Transcription vectors pJC213 (ATPase 8/6) and p2-6E (COII)(a gift of Dr. Philip Laipis) in bacterial strains TG-1 and JM109, respectively, were grown to log phase in Luria Broth (10gm NaCl, 10gm tryptone broth, and 5gm Yeast Extract per liter) pH 7.8, 0.5% glucose (w/v), and ampicillin (100μg/ml) at 37°C with shaking (6-8 hours). Chloramphenicol (20mg/liter) was then added and the incubation continued overnight. Plasmids were harvested using the Alkaline lysis method (described in Current Protocols in Molecular Biology, Ausubel, et al., 1987). The dried, recovered plasmid pellet was resuspended to 1mg/ml in doubly distilled, deionized water that was treated with diethylpyrocarbonate (DEPC). All water used in this and subsequent experiments was similarly prepared.

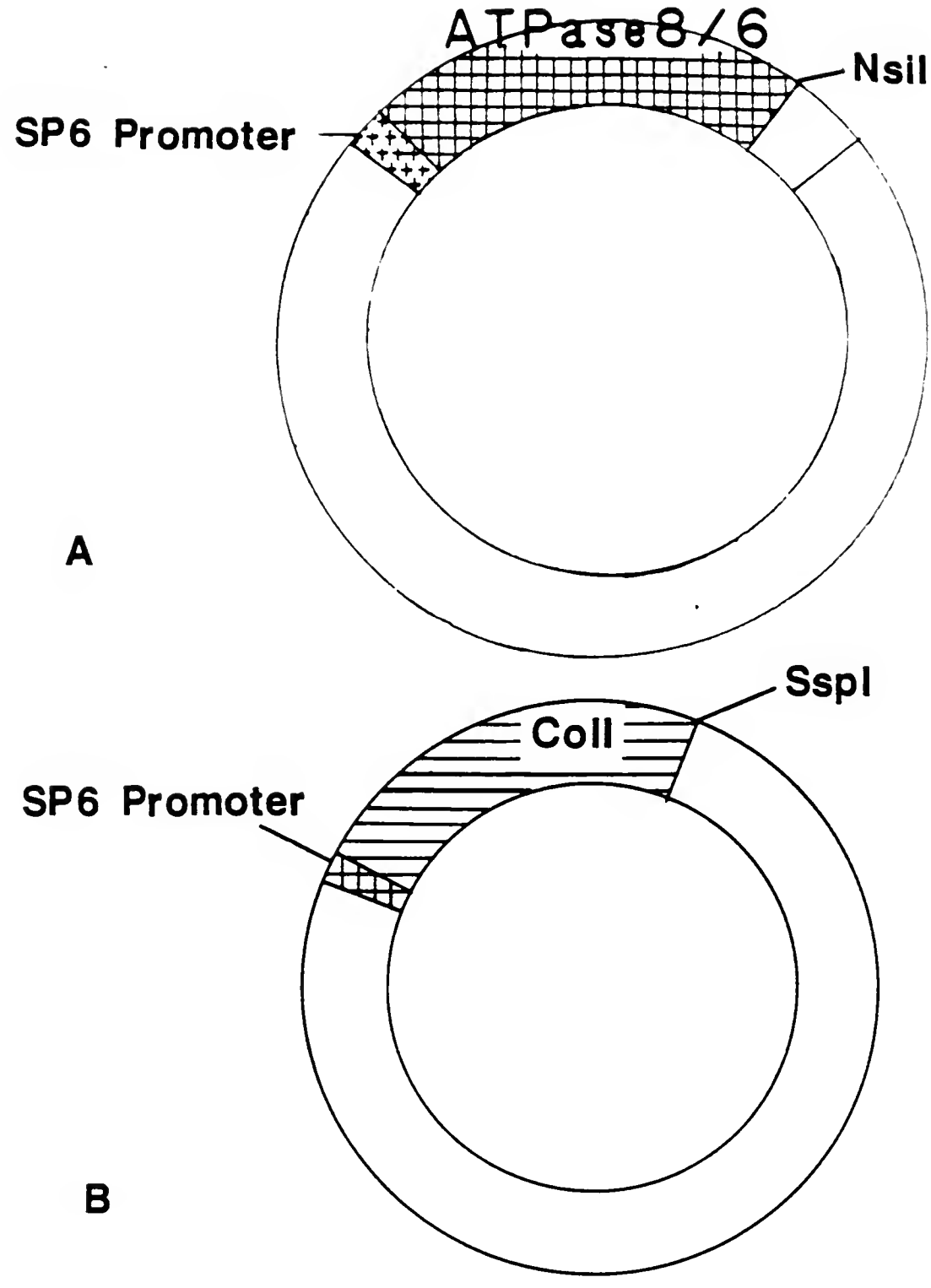


Figure 9. Transcription clones pJC213 (A8/6) and p2-6E (CoII). A) pJC213 for ATPase 8/6. B) p2-6E for COII (a gift of Dr. P. Laipis).

The pJC213 was then linearized with NsiI restriction endonuclease and p2-6E was linearized with SspI restriction endonuclease (Figure 9). Both vectors then were ethanol precipitated, pelleted, washed with 70% EtOH, dried, and resuspended to 0.2μg/1μl in water and stored at 4°C until needed.

Preparation of mRNA Transcripts

ATPase 8/6, Cytochrome Oxidase II, ATPase subunit β (the vector was the gift of Dr. A. Lewin), and poliovirus (the vector was the gift of Dr. Flanegan) mRNAs were synthesized by incubating 2μg of the templates for the genes mentioned above with 20-22.5 U SP6 RNA polymerase in 40mM Tris-HCl, 6mM $MgCl_2$, 2mM Spermidine-$(HCl)_3$, 10mM DTT, 100U RNAsin, 0.5mM ATP, 0.5mM CTP, 0.5mM GTP, and 0.5mM UTP, pH 7.9, for two hours at 37°C (Krieg and Melton, 1984). The synthesis reaction was incubated with 5 units of RNase-free DNaseI (RQ-1, Promega) for 30 minutes at 37°C. The resulting transcription products were analyzed by 1.5% agarose gel electrophoresis for reaction success, transcript size, and purity. Products were kept at 0°C and used within two hours.

Preparation of Radiolabelled Synthetic RNA

Labelled ATPase 8/6, COII, ATPase β subunit, and poliovirus mRNAs were synthesized by incubating 2μg of

appropriate template with 20-22.5 U SP6 RNA polymerase and 200μCi [α-^{32}P]CTP in 40mM Tris-HCl, 6mM $MgCl_2$, 2mM Spermidine-$(HCl)_3$, 10mM DTT, 50U RNAsin, 0.4mM ATP, 0.4mM CTP, 0.4mM GTP, and 0.4mM UTP, pH 7.9 for two hours at 37°C. The synthesis reaction was incubated with 5 units of RNase-free DNaseI for 30 minutes at 37°C. The transcription reaction was then run through Sephadex-50 RNase-free spin columns twice (Boehringer-Mannheim) equilibrated with 50/5 buffer (40mM KCl, 20mM HEPES-Cl, pH 7.3, 5mM MgOAc, and 6mM 2-mercaptoethanol) to remove free nucleotide (label) from solution. The transcripts were then quantified by their incorporation of radionucleotide with specific activities usually between 5-7x10^3CPM/pmoles. The resulting transcription products were analyzed by 1.5% agarose gel electrophoresis for success of the reaction, transcript size, and purity. Subsequent autoradiography of the gel confirmed that the removal of the labelled free-nucleotide had been removed. Products were kept at 0-4°C for use within two hours.

Preparation of 5' End Labelled Nucleic Acids

E. coli transfer RNA (tRNA)(Sigma), double stranded (dsDNA)(Promega), single stranded DNA (ssDNA)(TO 139), polyuridylic acid, poly(U), and polycytidylic acid, poly(C), (Sigma) were all labelled at the 5' end. Poly(U), poly(C) and ssDNA were labelled by incubating 100pmoles of the appropriate

template with 100pmoles of [γ-^{32}P]ATP and 10U of T4 polynucleotide kinase in 50mM Tris-Cl, pH 7.5, 10mM $MgCl_2$, 5mM DTT, and 0.1mM Spermidine for 20 minutes at 37°C. The other two templates were labelled using the Exchange Reaction (Perbal, 1988). Again 100pmoles of both template and [γ-^{32}P]ATP were incubated with 12U of T4 polynucleotide kinase and 0.25mM ADP in 50mM Immidazole-Cl, pH 7.5, 10mM $MgCl_2$, 5mM DTT, and 0.1mM Spermidine for 30 minutes at 37°C. Each reaction was spun through three separate sephadex G-50 spin columns to remove residual labelled nucleotide. Purity was tested by autoradiography after agarose gel electrophoresis. Greater than 95% of the free nucleotide was removed by each pass through the sephadex so that no residual nucleotide was detectable after the third column.

Binding Substrates to 28S Subunits

A number of substrates were bound to small subunits by incubating 1.0, 2.0, and 5.0pmoles (0.02, 0.04, or 0.1μM, respectively) 28S subunits with various concentrations of radiolabelled substrates from 0.02-1.0μM (1-50 pmoles) in 50μl of 50/5 buffer for 10 minutes at 35°C (Denslow et al., 1989; Liao and Spremulli, 1990b). The reactions were stopped by the addition of 800μl of ice cold 50/5 buffer.

Similarly, 9 pmoles (0.18μM) of radiolabelled COII or ATPase 8/6 mRNA was incubated with 4.5-90 pmoles (0.09-1.8μM)

small subunits (native and derived) in 50/5 buffer for 10 minutes at 35°C in a reaction volume of 50μl. The reactions were stopped by the addition of 800μl of ice cold 50/5 buffer.

Competition studies were carried out by mixing a saturating amount of radiolabelled COII mRNA with varying amounts of competitor prior to incubation with 1.0 or 5.0 pmoles (0.02 or 0.1μM) of 28S subunits in 50/5 buffer for 10 minutes at 35°C in a reaction volume of 50μl. The reactions were stopped by the addition of 800μl of ice cold 50/5 buffer.

Millipore Filter Binding Assay

After binding the experimental substrate(s), the stopped reaction mixture was mixed gently on a vortex mixer and the ribosomes were adsorbed to wettable 0.45μm HAWP type millipore filters. The filters were previously rinsed in 5 ml of 50/5 buffer to remove all glycerol. The tubes were rinsed and vortexed with 800μl of ice cold 50/5 buffer, which was then applied to the filters and the filters were washed twice with 8-10 ml of ice cold 50/5 buffer. Washed filters were immersed in scintillant (ScintiVerse II, Sigma) and counted on a Beckman LS 380 scintillation counter (Denslow et al., 1991).

Sucrose Density Gradient Centrifugation

Sucrose density gradients were employed to determine the stoichiometry of 28S subunit/mRNA binding interactions. After

binding reactions were completed they were loaded onto 10-30% sucrose density gradients in 50/5 buffer. These gradients were formed in SW 27 cellulose nitrate (wettable) tubes using a Beckman gradient former. Loading of the stopped reactions was accomplished by layering up to 2 ml on top of the gradients. Tubes were placed in an SW 27 rotor and centrifuged for 18 hours at 23,000 rpm at 4°C.

The gradients were then monitored and fractionated using a Pharmacia FPLC System, UV detector, chart recorder, and fraction collector. To the collected fractions (2 ml) 2 ml of ScintiVerse II (Sigma) scintillation cocktail was added and the fractions were then counted on a Beckman LS380 scintillation counter.

Chemical Modifications and Enzymatic Cleavages

COII mRNA (9-10 pmoles, 180-200nM) was diluted in 50/5 buffer to 50μl and modified at 35°C for 10 minutes by the addition of RNase A(10^{-2}-10^{-4}U/μg RNA), or RNase T_1 (10^{-2}-10^{-4}U/μg RNA), or RNase V_1 (0.45-10^{-3}U/μg RNA). DMS modifications were carried out under the same conditions but with 1-2μl of DMS being added, gently mixed, and incubated at 35°C for 0.5 to 2 minutes. Modifications and cleavages were done in the presence and absence of 18-20pmoles (360-400nM) of 28S subunits. The reactions were stopped by the addition of 150μl

of ice cold stop buffer (100mM NaAc, 3.3mM EDTA, and 120 mg/ml yeast tRNA).

The enzymatic reactions were then extracted twice with phenol that was equilibrated with HE (10mM HEPES-Cl pH 7.3 and 1mM EDTA), and once with chloroform. The mRNA was precipitated by addition of 2.5 volumes of cold absolute ethanol followed by incubation at -70°C for 60 minutes. The mRNA was recovered by centrifugation at 15,000 rpm in a Hermle microfuge, washed with 70% ethanol, and dried in a Savant Speedvac. The mRNA was resuspended in water to 0.1 μM and stored at -70°C until needed.

DMS reactions were immediately precipitated by addition of 2.5 volumes of cold absolute ethanol and kept at -70°C for 60 minutes. The mRNA was recovered by centrifugation at 15,000 rpm in a Hermle microfuge, washed with 70% ethanol, and dried in a Savant Speedvac. The mRNA was resuspended in 200μl HE and then extracted twice with phenol that was equilibrated with HE, and once with chloroform. The RNA was recovered by centrifugation, washed with 70% ethanol, and dried. The RNA was resuspended in water to 22μg/ml (0.1μM) and stored at -70°C until needed.

All reactions were carried out in triplicate. In addition, each nuclease and DMS were titrated so that approximately one chemical modification or one RNase cleavage would occur every 150 bases to provide optimal analysis (Figure 10).

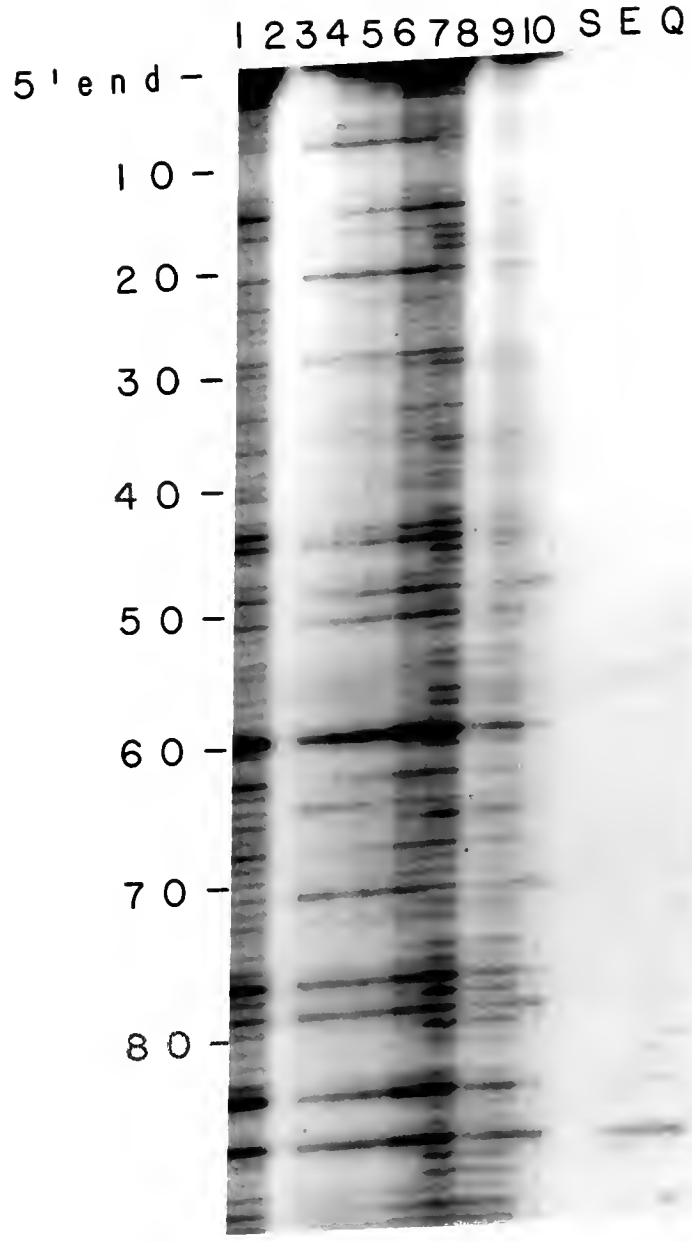


Figure 10. Titration of RNase A and DMS Modifications of CoII mRNA (Primer extension Ω). Lane 1: Control- no RNAase A; lanes 2-6: $1x10^{-2}$, 7.5, 5, 2.5, and $1x10^{-3}$U RNase A, respectively; lanes 7 and 9: 1μl of DMS incubated for 1 and 2 minutes, respectively; lanes 8 and 10: 2μl of DMS incubated for 1 and 2 minutes, respectively; and lanes 11-13: Sequence (A,C,G).

DNA Primers

Five DNA primers were designed to be complementary to the COII mRNA, each 17 nucleotides long (Table 2). Each sequence was specific for a unique area on the mRNA (i.e., recognized for annealing to a particular mRNA sequence and no others in that mRNA or the 12S rRNA) and designed to be separated by 150-200 nucleotide intervals (Figure 11), about the distance that can be easily analyzed on the denaturing gels used. Particular care was taken so that the 3' end of the oligonucleotides would give efficient priming without promoting spurious annealing. The guanosine and cytosine content of these oligonucleotides was also considered, to maintain the G+C content at about 50%, if practical. The oligonucleotide primers were synthesized using an Applied Biosystems Model 380B synthesizer in the University of Florida ICBR DNA Synthesis Core Facility.

Extension of Oligodeoxyribonucleotide Primers

Extensions were initiated by incubating 25 pmoles (500nM) of the appropriate primers with 1.0 pmoles of the mRNA (20nM) in 50mM KCl, 9mM $MgCl_2$, 20mM 2-mercaptoethanol, 50mM Tris-HCl, 25μM dATP, 250μM dCTP, 250μM dGTP, 250μM dTTP, and 10-15μCi [α-^{32}P]dATP (>600 Ci/mmol) in a final reaction volume of

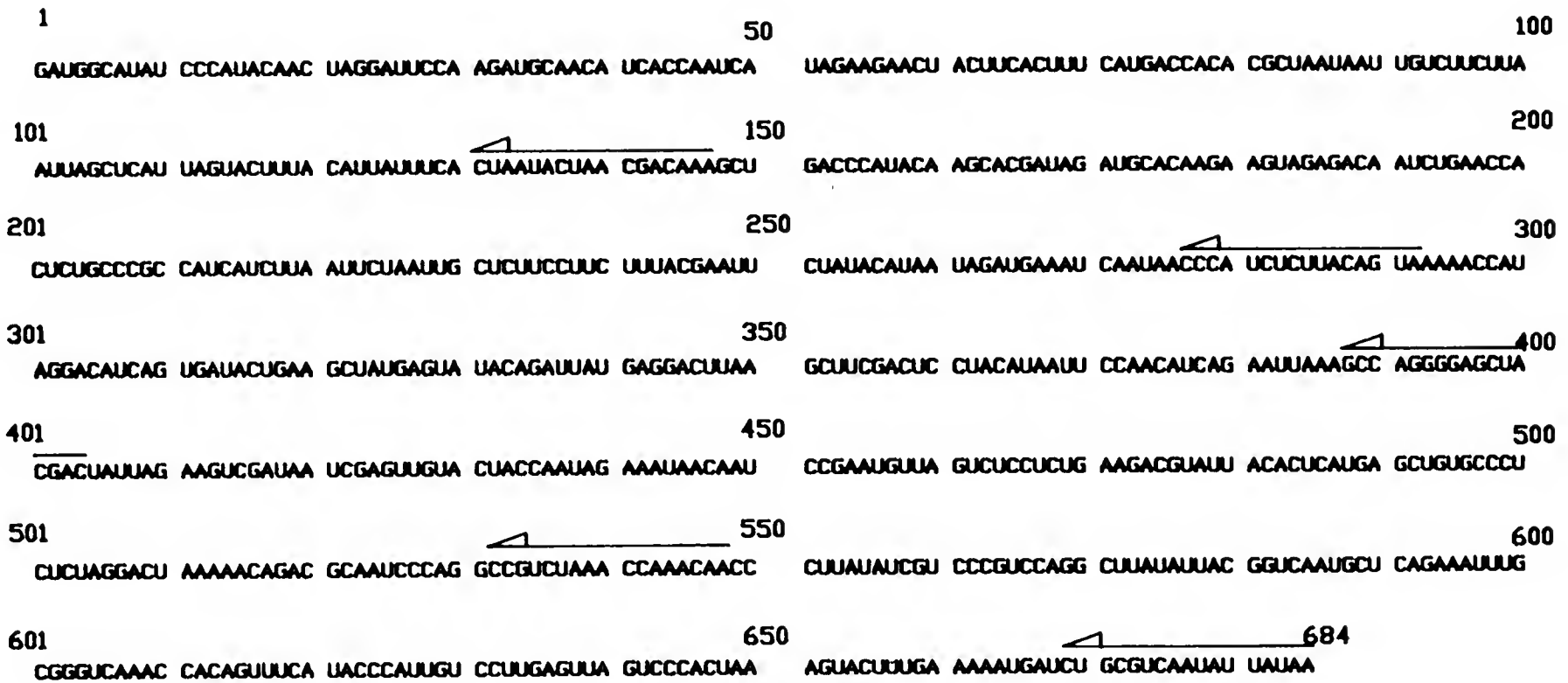


Figure 11. The Location of Primers on the COII mRNA Primary Structure. Primers are from 5' end: Omega (Ω), Alpha (α), Beta (β), Gamma (ɣ), and Delta (δ).

50μl at 65°C for 5 minutes. The mixture was then placed into a 50°C water bath to allow annealing to occur. The primers were then extended by adding 10 U AMV reverse transcriptase to the reaction mix, and incubating at 50°C for 30 minutes.

Primer extension reactions were stopped by the addition of 150μl of ice cold reverse transcription stop solution (100mM NaAc, 6.6mM EDTA, 0.1μg/μl carrier yeast tRNA, and 90% EtOH). The cDNA reverse transcription products were then kept at -70°C for 60 minutes, and the cDNA was recovered by centrifugation at 15,000 rpm in a Hermle microfuge, washed with 70% EtOH, dried, and resuspended in 20μl loading solution (583 mg/ml bromophenol blue, 583 mg/ml xylene cyanol, 8.3mM EDTA, and 10.8M formamide. Samples were heated for 3 minutes at 90°C and quenched on ice before loading onto gels.

Denaturing Gels

Denaturing gels (6% acrylamide, 0.3% bisacrylamide, 8 M urea) were formed as 3:1 wedges (to afford better linear separation) and were run at 50°C. The gels were run in TBE buffer (10mM Tris, 10mM Boric Acid, and 0.1mM EDTA, pH 7.8) at 80 watts for approximately 3 hours. These gels were then adhered to Whatman 3MM chromatography paper and dried on an Ephotec gel drier under vacuum at 80°C for 20 minutes. The dried gels were then placed in autoradiographic cassettes with Kodak XAR X-ray film and exposed at least 15 hours.

Gel Reading Algorithm

In order to determine whether a given nucleotide was modified, its autoradiographic band intensity was compared to the same band region in the control gel. In addition, the intensity of the experimental band was compared to background bands within in the same lane (this method helped offset the problems of non-uniform background from lane to lane and from top to bottom on the gel). Thus, if the band for a given nucleotide was darker than the control and greater in intensity relative to the background within the lane, that nucleotide was scored as accessible. Occasionally, a band would be observed even though no modifier was added. This occurrence could be explained by a number of phenomena, including false priming of the carrier yeast tRNA by the primers for COII mRNA, strong secondary structural effects of the COII mRNA, false priming of 12S rRNA (when present) by the primers to COII mRNA, and susceptibility to RNases not purified from the isolated 28S subunits. The priming and extension of yeast tRNA were tested, and no evidence of extensions was apparent. The priming of 12S rRNA was also tested with only a few faint bands being seen in a light background smear. The bands were usually in between the "normal" cDNA banding pattern. In addition, all data were first recorded against the primary structure so as not to be prejudiced by the predicted secondary structure.

Scanning Densitometry

Scanning densitometry was done on the General Imaging Scanner of the Protein Core Facility, ICBR, to quantify the data from the autoradiography of denaturing gels. This proved particular useful in determining the extent of protection afforded by small subunit interactions.

NUCLEIC ACID BINDING PROPERTIES OF THE SMALL SUBUNIT OF BOVINE MITOCHONDRIAL RIBOSOMES

Significance of 28S Ribosomal Subunit Binding Properties

Bovine mitochondrial ribosomes were chosen as a model system to address a number of questions unique to the mammalian mitochondrial translation system. These ribosomes contain nearly twice as much protein mass as the *E. coli* ribosomes, roughly half as much RNA, and still retain similar dimensions and appearance of the latter upon electron micrographic inspection (Hamilton and O'Brien, 1974, Lake *et al.*, 1976). It is, however, the ability of these ribosomes to bind the unusual mRNAs of the mitochondrion that is of particular interest to this work. Where the interaction occurs on the mRNA, and what the order of assembly and requirements for additional factors might be remain to be answered. At the outset it must be remembered that no *in vitro* protein synthetic system is available for the mitochondrion such as is commonly used in the other systems (e.g., reticulocyte lysates for eukaryotic systems).

The mitochondrial mRNAs have little or no 5' leader and no 3' untranslated sequence, though they are polyadenylated. They are not capped at their 5' end (Gaines,

et al., 1988), like most eukaryotic mRNAs, nor do they possess a S-D like sequence. They are produced as a single transcript (>16 kb) of the entire heavy strand of the mitochondrial genome, which is composed of 12 intronless open reading frames (all coding for inner mitochondrial membrane proteins) and punctuated by 22 tRNAs which serve as processing signals. The light strand of the mitochondrial genome encodes seven tRNAs and one protein product, ND6, that shares all other mitochondrial mRNA traits, including being transcribed as part of single, full length polycistron. Additionally, the rRNAs are transcribed in a separate event that excludes all mRNAs and they have been quantitated at 100 fold excess over mRNAs in HeLa cell mitochondria (Gaines and Attardi, 1984) and at a >10 fold excess over mRNAs in adult rat liver mitochondria (Cantatore,et al.,1984).

The small subunit is capable of binding these mRNA molecules through a novel and yet to be defined mechanism. Speculation has suggested the presence of an internal S-D like sequence may be important for the binding of some of these mRNAs but not all (Saccone et al., 1985); yet this region on the 12S rRNA proved inaccessible to probing in the subunit (Faunce and O'Brien, manuscript in process). Therefore, it is less likely to be able to form base pairs with the mRNAs. Another speculation proposes that the 5' ends of the mRNAs may be important (Denslow et al., 1989). The 28S ribosome may recognize a particular secondary structure in the mRNA which

may be sufficient for the binding of mRNA by the ribosome. A third possibility for the mechanism by which the mitochondrial ribosome binds mRNA is the presence of a sequence nonspecific RNA binding site on the mitochondrial ribosome (Denslow et al., 1989). The roughly thirty bases long binding domain of this binding site was disclosed by studies binding oligoribonucleotides of various lengths. Furthermore, an RNase T_1 protection domain of up to eighty bases in length was provided on bound RNA (5' end labelled). The use of binding assays should provide some insight into the interaction between mitochondrial 28S subunits and mRNAs.

Bovine Mitochondrial mRNA Binding Properties of 28S Ribosomal Subunits

In order to determine the affinity and stoichiometry of 28S subunit binding to mitochondrial mRNAs Millipore filter binding assays and sucrose density gradients were used. The binding conditions closely simulated those previously used successfully (Denslow et al., 1989; Liao and Spremulli, 1990b). Initially, both native and derived 28S subunits (see Material and Methods) were used to see whether any differences in their ability to bind message were readily apparent. The results are displayed as the mean of three experiments in which the ATPase 8/6 and COII mRNAs were incubated 28S subunits, native or derived. The reaction data were plotted with the aid of a personal computer and Sigmaplot 4.1, and

represented as pmoles of radiolabelled message bound versus pmoles of 28S subunits.

The apparent K_d and stoichiometry data were then calculated by Scatchard analysis (Scatchard, 1949) using the following equation:

$$r/C = -1/K_d\ (r) + n/K_d$$

where: r= pmoles 28S bound/pmoles mRNA; C= concentration of free ligand (28S ribs); K_d= dissociation constant; and n=number of binding sites.

Figure 12 shows the plot of the experimental data and Table 3 shows the results of the subsequent Scatchard analysis. Both native and derived subunits bind mitochondrial mRNA with similar affinity and stoichiometry. Analysis indicates that each subunit has a single binding site, and each type of subunit binds the mRNA with the same affinity (K_d-3-5x10^{-8}M). Maximum binding of the mRNAs occurred at a ribosome to message ratio greater than 2:1 with approximately 85-90% of the message being bound. Henceforth the 28S subunits referred to in this work will be derived 28S subunits due to their enhanced purity by a second sucrose density gradient, their equivalent binding affinity to native subunits, and the relative availability of derived subunits.

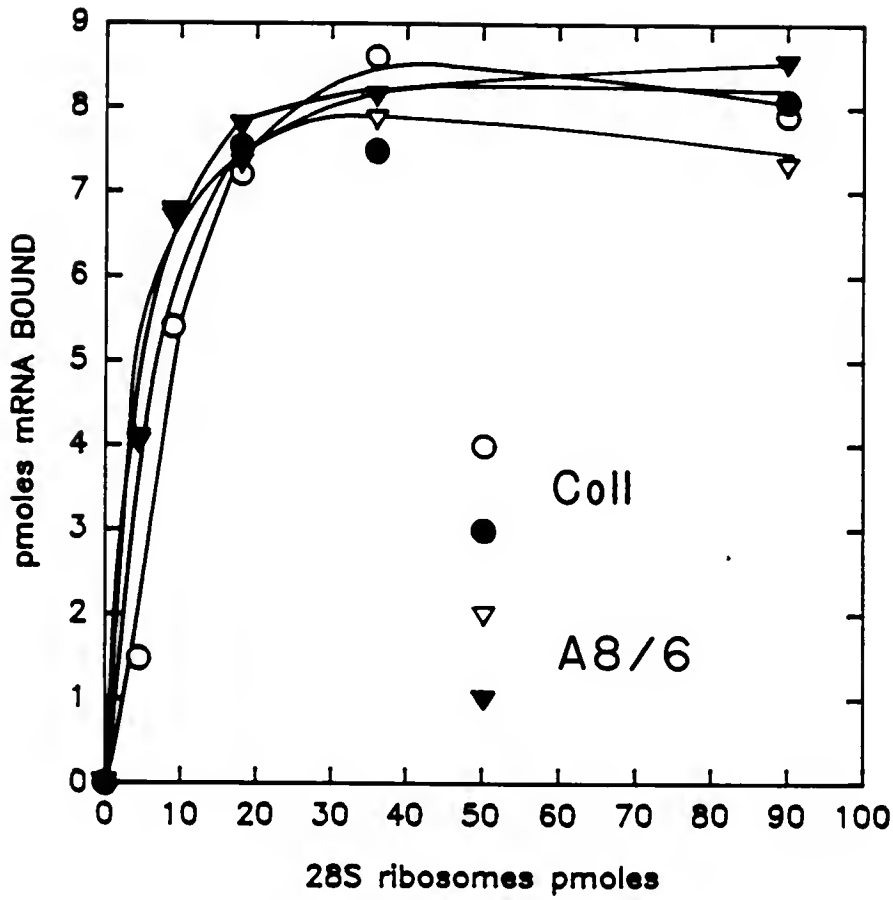


Figure 12. Binding of Mitochondrial mRNAs to 28S Subunits. Incubations of 9 pmoles (0.18μM) mitochondrial mRNA, both CoII and ATPase 8/6 mRNAs with 4.5-90 pmoles (0.09-1.8μM) 28S subunits (native and derived) were carried out in a 50μl reaction volume at 35°C. The reactions were stopped after 10 minutes by the addition of 800μl of ice cold 50/5 buffer. Binding was assayed by Millipore filter binding. Each data point represents the mean of three replicate reactions. Circles represent CoII mRNA, triangles are ATPase 8/6 mRNA, open symbols represent native 28S subunits, and closed symbols derived 28S subunits.

Table 3

Binding Analysis of Mitochondrial mRNAs to 28S Subunits

Mitochondrial Messenger RNA	28S Subunit Preparation Type	Affinity $K_d x 10^{-9} M \pm sd$	Sites on 28S Subunits $n \pm sd$
ATPase 8/6	Native	29.7±2.4	0.88±0.10
	Derived	28.0±7.0	0.96±0.13
COII	Native	52.8±3.2	1.00±0.06
	Derived	51.3±2.3	1.02±0.10

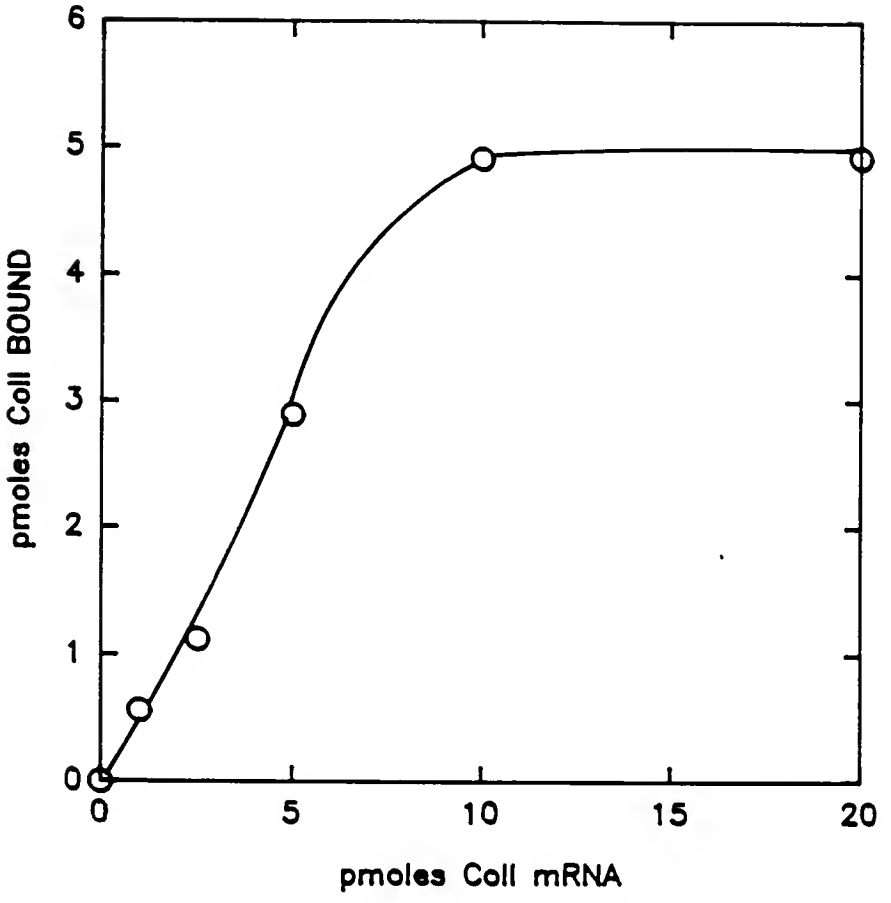


Figure 13. Binding of CoII mRNA to 28S Subunits. A reaction mixture of 5.0 pmoles (0.1μM) 28S subunits and 1.0-20.0 pmoles (0.02-0.4μM) CoII mRNA was incubated for 10 minutes at 35°C and assayed by Millipore filter binding as previously described.

Table 4

Binding Analysis of COII mRNA to 28S Subunits

Messenger RNA	Affinity $K_d x10^{-9} M \pm sd$	Sites on 28S Subunits $n \pm sd$
COII	56±10	1.3±0.07

Binding was also done holding 28S subunits constant at concentrations of 0.04 or 0.1μM and varying the mRNA concentration from 0.02-0.4μM. The same equations were used for analysis of binding and the resulting plot and analysis are displayed in Figure 13 and Table 4. These data verified the previous binding results so the 0.1μM concentration of ribosomes was used for most of the subsequent binding experiments. The apparent K_d for the binding of mitochondrial mRNAs was approximately 30-50nM and the saturation of 28S subunits was reached at a ratio of 1.5-2 mRNAs to small ribosomal subunits. Again a single binding site on the ribosome was resolved.

The ability of one mRNA to bind to one small subunit did not rule out the possibility of a single message providing multiple binding sites for ribosomes when ribosomes were in excess. Millipore filter assays were limited for determining this because they adsorb the 28S subunit whether or not it has a bound mRNA. Sucrose density gradients were decidedly the fastest and most practical method for addressing this question (Denslow and O'Brien personal communication). The gradients were prepared as they were for the isolation of derived 28S from 39S subunits except that the 50/5 binding buffer was used instead of the 300/5 buffer, as described in Materials and Methods. These conditions have been used to discriminate between 28S monomers and "dimers" of the 28S subunits (Denslow, personal communication). Here 28S subunits

(9-72 pmoles) were incubated with 9 pmoles of [^{32}P] radiolabelled COII mRNA, prepared as described in the Materials and Methods section, for 10 minutes at 35°C in a final volume of 50μl. The reaction was stopped by adding 1ml of ice cold 50/5 buffer to the reaction mixture. Two 1ml reactions were loaded onto the gradient for centrifugation. These gradients were then monitored and fractionated (Materials and Methods).

Fractions of 2 ml were collected and subsequently counted in a Beckman LS 380 scintillation counter to determine where the radiolabelled mRNA had sedimented. For the results of these gradients see Figure 14. The binding data has been plotted in Figure 15 and the analysis recorded in Table 5. The ribosome sedimentation profiles with the radioactivity profile representing the labelled COII message, demonstrating that the mRNA accompanies the 28S ribosome. Control gradients (Figure 14, A) lacking ribosomes, show the mRNA sedimenting at an approximately 4S peak, near the top of the gradient. Incubation in the presence of 28S subunits (Figure 14,C) results in bound mRNA sedimenting with the 28S subunits. This observation continues for incubations with increased amounts of 28S subunits (Figure 14,D-F) and the amount of mRNA bound plateaus around the 2:1 ribosome to message ratio (Figure 15). Significantly, no radioactivity (mRNA) appears in the position expected for 28S dimer-mRNA complexes (approximately the 35S position in the gradient).

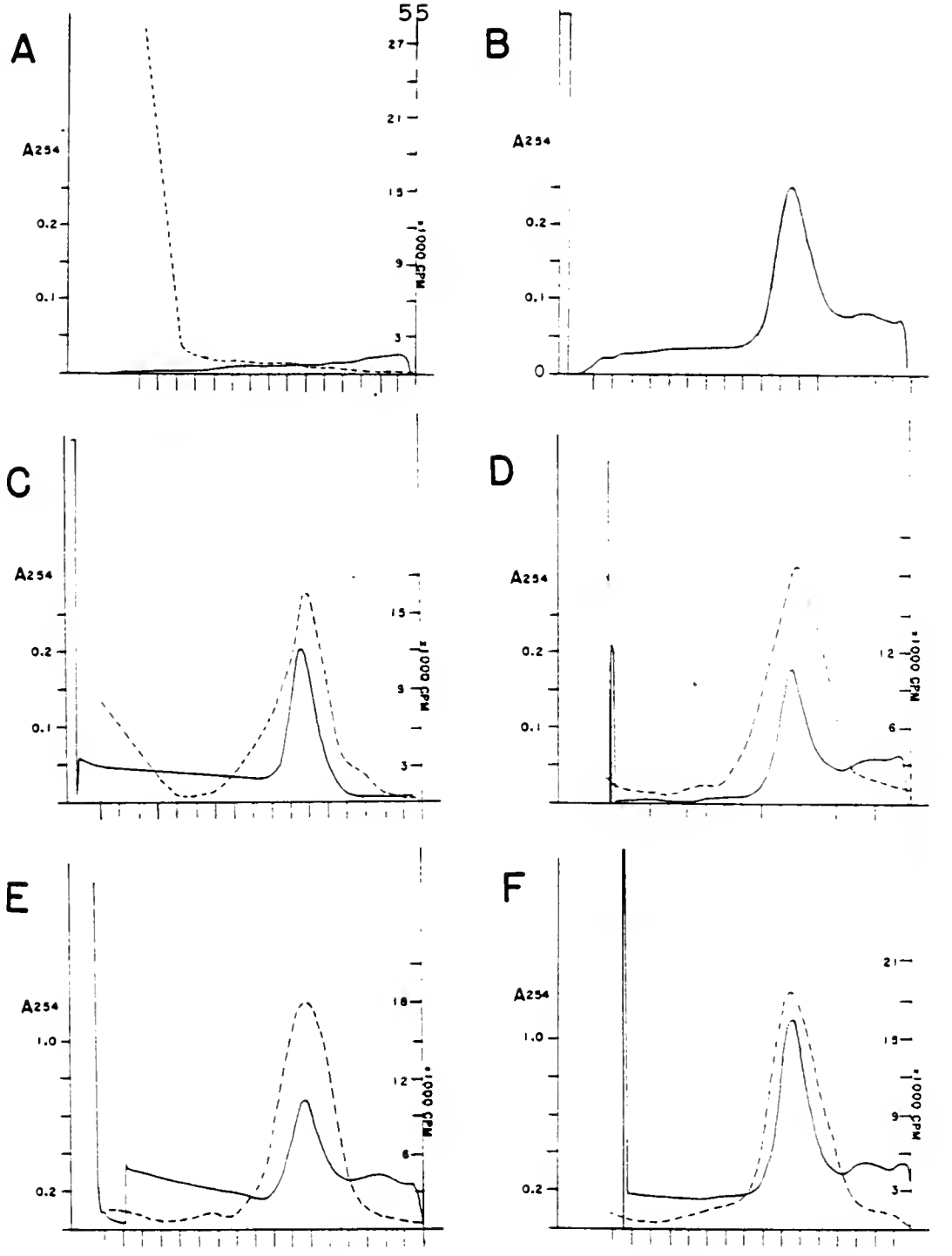


Figure 14. Sucrose Density Gradients of CoII mRNA to 28S subunit Binding. The mRNAs specific activity was 7200 CPM/pmole. A: mRNA-no ribosomes; B: Ribosomes-no mRNA; C-F: 1:1, 2:1, 4:1, and 8:1 ratio of ribosomes to mRNA concentrations, respectively.

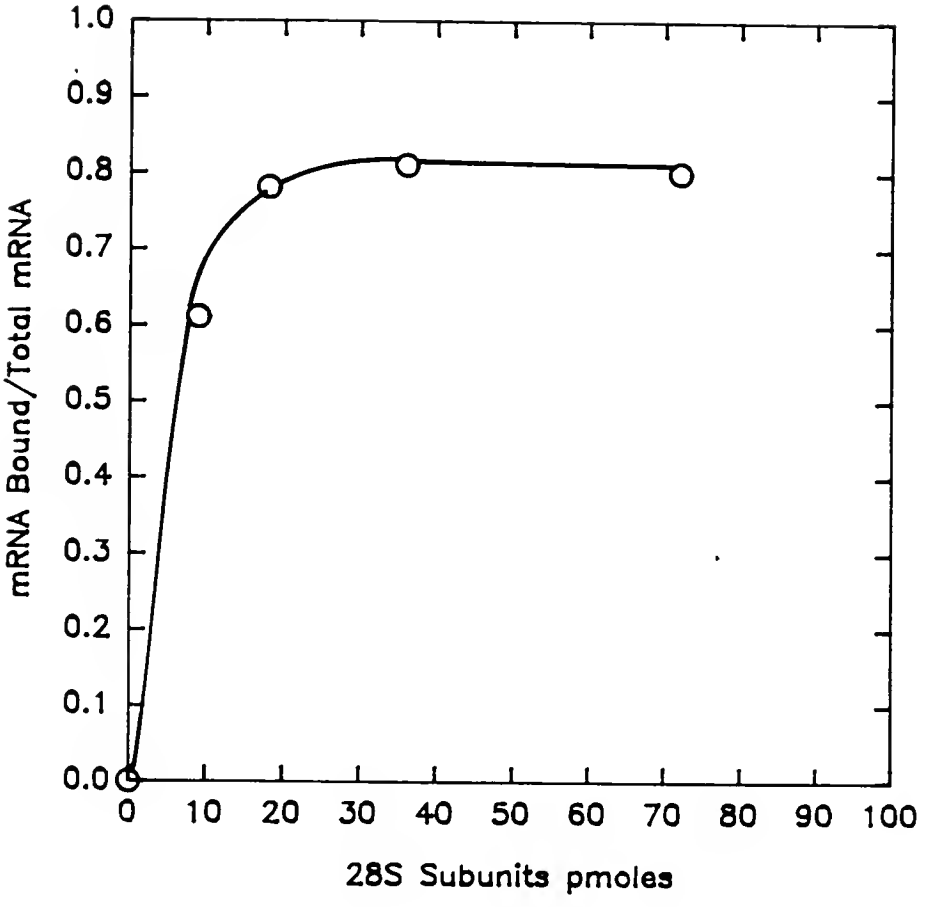


Figure 15. Plot of CoII mRNA Binding Assayed by Sucrose Density Gradients. 18 pmoles of CoII mRNA incubated with 0, 18, 36, 72, 144 pmoles of 28S subunits, as indicated, prior to analysis of the binding reactions by sucrose density gradient centrifugation (Materials and Methods).

Table 5

Binding Analysis of Sucrose Density Gradients

Fig 14	28S Conc μM	28S SDG pmol	28S peak pmol	28S recov %	mRNA Conc μM	mRNA SDG pmol	mRNA recov %	mRNA 28S peak pmol	mRNA Not Bound pmol	mRNA Bound % of Total
A	0.0	0.0	----	----	0.18	18	43.6	----	7.85	----
B	0.36	36	17.1	47.5	0.0	0.0	----	----	----	----
C	0.18	18	9.8	54.4	0.18	18	56.8	6.2	4.0	60.7
D	0.36	36	17.0	47.2	0.18	18	63.3	8.9	2.5	78.1
E	0.72	72	40.4	56.1	0.18	18	59.7	8.67	2.03	81.0
F	1.44	144	79.2	55.0	0.18	18	58.5	8.43	2.07	80.0

Therefore, there is no evidence for two subunits binding to a single mRNA (even at large excess of ribosomes over message), and there appears to be only one ribosome binding site on the mRNA.

On the basis of the analysis of mRNA binding, assayed by Millipore filter binding, it can be stated that 28S subunits bind each of these two mitochondrial messages (COII and ATPase 8/6) with essentially the same affinity (apparent Kd of about $3\text{-}5\text{x}10^{-8}$M). To ensure that only one ribosome was interacting with the message, conditions were used in subsequent experiments (i.e., footprinting), that result in approximately 80% saturation of the mRNA (9 pmoles mRNA and 18 pmoles of 28S subunits). The mRNA binding site on the ribosome could be saturated at 1.5-2 messages per 28S concentration. Finally, from the observations of mRNA binding assayed by sucrose density gradient centrifugation it can be said that 28S subunits bind COII mRNA with unit stoichiometry: one mRNA molecule per 28S subunit (Table 5) and reaches maximum binding at about the previously determined 2:1 ribosome to message ratio.

Interaction of mRNAs of Non-Mitochondrial Origin with Mitochondrial 28S Subunits

Given the ability of 28S ribosomes to bind mitochondrial mRNAs, it is of interest to consider whether mitochondrial ribosomes can bind other (non-mitochondrial) kinds of mRNAs --

or is there something "special" about mt mRNAs that promotes their initiation (and not that of non-mt mRNAs)? Four non-mitochondrial mRNAs were chosen to examine this question.

Poly(U) and poly(C) are homopolymers of ribonucleotides uridylic and cytidylic acid, respectively. Poly(U) has been used for investigations of ribosome-RNA interaction studies in all systems (Denslow et al., 1989; Kumazawa et al., 1991; Hill et al., 1990) and here represents an essentially unstructured (i.e., single stranded) mRNA. Poly(C) falls into a similar category but may have some ability to form an A-type helix even in the single stranded form, expected in the conditions used here (Saenger, 1985). A-type helices are common to most RNAs, whether single or double stranded, because of the "$C_{3'}$-endo pucker" of the ribose ring which brings the $O_{3'}$-phosphate of the adjacent nucleotide closer to its own 5'-phosphate. Poly(C) helical structure differs from the common A-RNA form in that it displays a six base per turn conformation and its "single helices retain standard nucleotide conformation even better than the stereochemically more demanding double helices" (Figure 16). So, poly(C) as a single stranded template, is expected to exhibit structural features which differ from poly(U).

Perhaps 28S subunits will display a binding affinity higher for unstructured single-stranded templates like poly(U) than for mitochondrial messages. If not, then possibly the mt mRNAs have a sequence or structural feature that may be important for 28S subunit binding. Also, a difference in

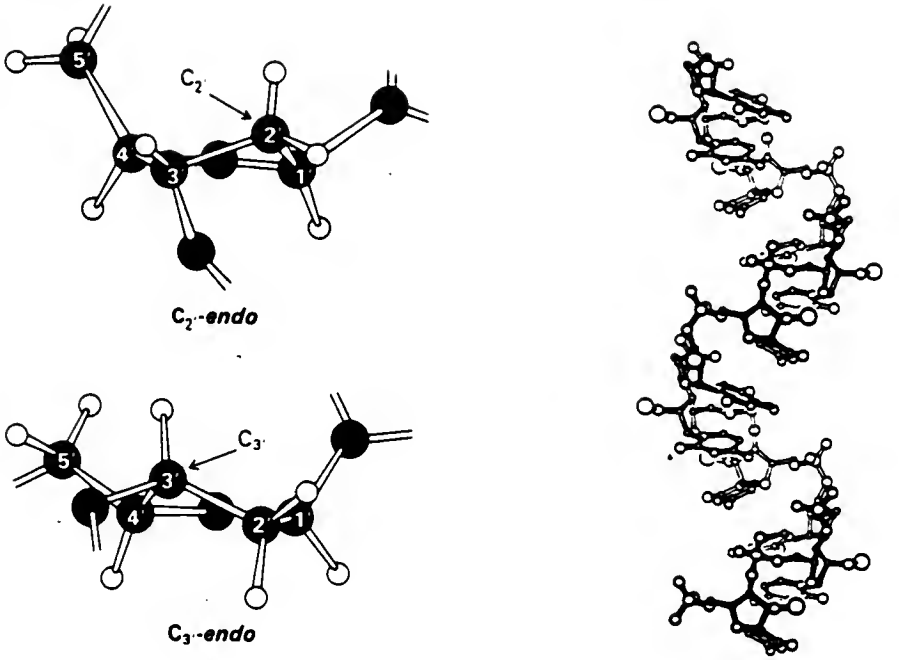


Figure 16. $C_{3'}$-endo 'Pucker' of Ribonucleotides (Stryer et al., 1988) and Structure of Poly(C) Single Strand Helix (Saenger, 1985).

the binding properties of poly(U) and poly(C) may indicate the effects of an alternate helical structure on binding of templates to mitochondrial ribosomes.

Additionally, two non-mitochondrial but naturally occurring messages, ATPase β subunit from E. coli and poliovirus mRNAs, were chosen as well. Expression vectors for these mRNAs were provided by Drs. A. Lewin and J. Flanegan, respectively. These two non-mitochondrial messages would furnish a basis for comparing their binding to that of the mt mRNAs and for answering the question of whether non-mitochondrial messages bind differently (or at all). All four messages together should provide clues into the mRNA features required to promote/permit binding.

These various mRNAs were incubated with 28S subunits in the same fashion as COII and ATPase 8/6 messages, 1-20 pmoles of mRNA with 5 pmoles of 28S subunits. The data were plotted and analyzed as above (Figure 17 and Table 6). Again a single binding site on the ribosome was exhibited, just as for the mitochondrial mRNAs tested. Furthermore, these non-mitochondrial mRNAs had a similar affinity for the 28S subunit ($5x10^{-8}M$) with the exception of the unusually structured poly(C)($10^{-7}M$). Thus, the 28S subunit appears to have a general mRNA binding site which can accommodate these and probably other messages.

Poly(U) binding by 28S subunits was also analyzed by sucrose density gradient centrifugation to determine whether

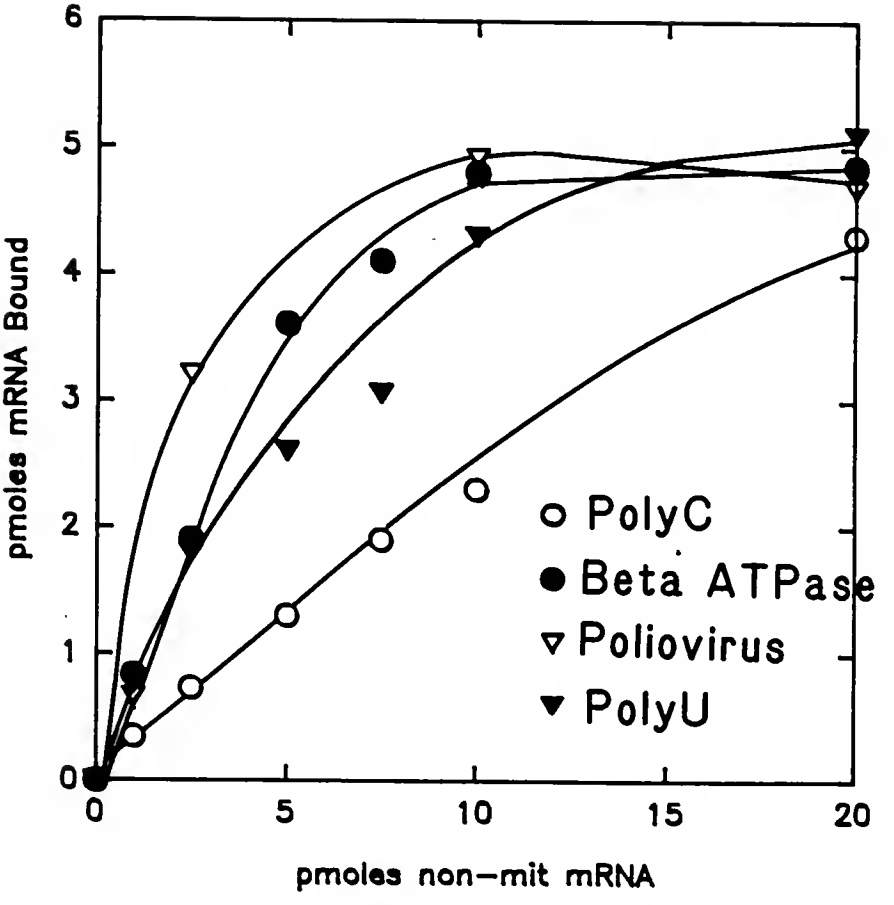


Figure 17. Binding of Non-mitochondrial mRNAs by 28S Subunits. 1-20 pmoles (0.02-0.40μM) mRNA were incubated with 5 pmoles (0.10μM) of 28S subunits for 10 minutes at 35°C in a 50μl final volume.

Table 6

Binding Analysis of Non-mitochondrial mRNAs.

Messenger RNA	Affinity $K_d x 10^{-9} M \pm sd$	Sites on 28S Subunits n±sd
β ATPase	34.5±8.4	1.26±0.12
Poliovirus	32.2±4.8	1.18±0.04
Poly(U)	46.4±7.5	1.03±0.20
Poly(C)	136±32	0.82±0.22

more than one subunit could bind to extended, "unstructured" mRNA. The resulting collection of gradients are recorded in Figure 18. Figure 18 (B and C) shows that incubation of 9 pmoles (0.18μM) poly(U) with either 18 pmoles (0.36μM) or 36 pmoles (0.72μM) of 28S subunits resulted only in poly(U) sedimenting in the 28S peak. The lack of entities sedimenting at values greater than 28S, where dimers of 28S would sediment, showed that only one small subunit was bound per poly(U). Table 7 shows that of the poly(U) in the gradient greater than 80% was bound by the 28S subunits under conditions where the 28S subunits concentrations were 2 and 4 fold higher than the RNA. This is similar to the binding seen for COII mRNA under SDG binding conditions (Table 5). An approximated K_d of 34.5nM can be calculated.

This template RNA, poly(U), and a bovine mitochondrial mRNA are binding in similar fashion under similar conditions, which lends some credence to the possibility of the binding being functional (poly-Phe has been translated). Neither COII mRNA nor poly(U) were capable of being bound by more than one 28S subunit under these conditions. It would seem that the 28S subunit may preclude other messages from binding. Perhaps in the case of an unstructured RNA like poly(U) the molecule is wrapped around the ribosome. This could be the case for all RNAs or the structure of mRNAs might limit their availability for binding. If a message was folded back on itself, "knotted", in its tertiary form, it could potentially

shield sequences that might otherwise be bound by the ribosome's 30 base binding domain.

Interaction of Non-message Polynucleotides with Mitochondrial 28S Subunits

What the parameters are for the binding of a polynucleotide by a 28S subunit cannot be defined by the previous attempts. So several other non-message polynucleotides were employed under the now standard incubation conditions to determine what characteristics of a polynucleotide might allow or prevent a ribosome from associating with it. A double stranded DNA (3200 base pairs)(linearized, pGEM -3zf, Promega), a single stranded DNA (TO 139)(confirmed as ssDNA by OLIGO 4.0 Primer Analysis Software, Rychlik, 1989), tRNA (E.coli tRNA, Sigma), as an example of a highly structured RNA molecule normally interacting with ribosomes (Figure 19) were used in Millipore filter assays of 28S subunits. All polynucleotides were 5' end labelled and all exceeded the minimum polynucleotide length requirement of 18 bases, previously determined for efficient binding of poly(U)(O'Brien, et al., 1990).

Small subunits were maintained at a concentration of 0.1μM while substrate concentrations were varied from 0.04 to 0.8μM. The results of the Millipore filter assays are shown in Figure 20 and the analysis of the binding is displayed in Table 8. Even at 20-fold excess of polynucleotide to 28S

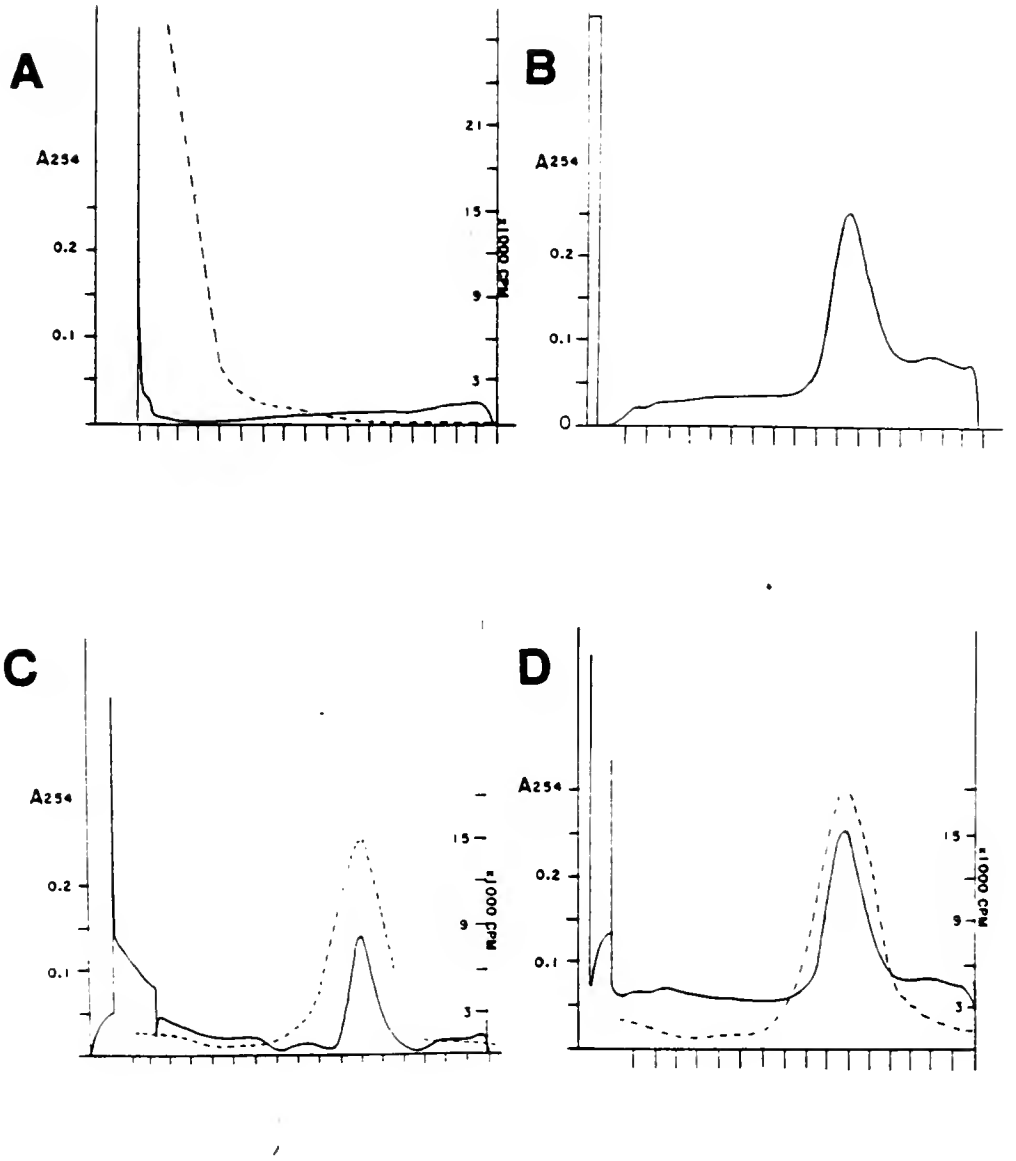
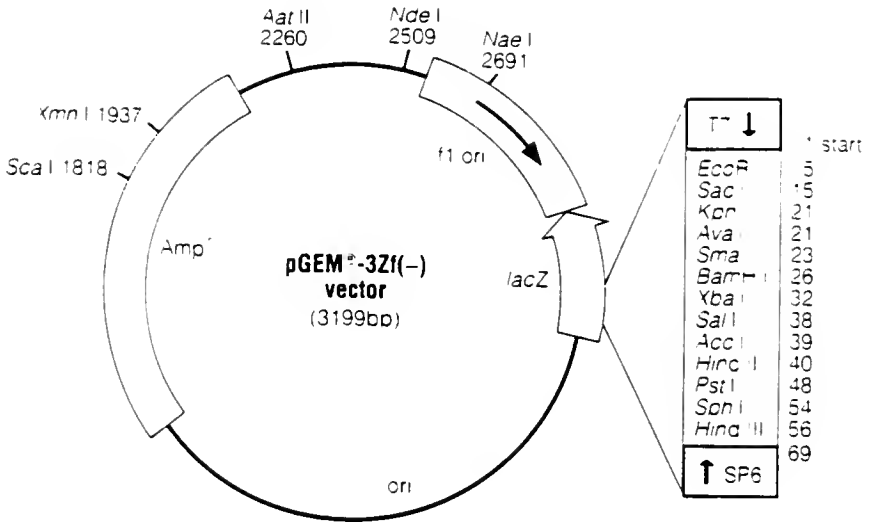


Figure 18. Sucrose Density Gradient with Poly(U). A: Poly(U) alone; B: 28S subunits alone; C: 2:1 28S subunits to poly(U); and D: 4:1 28S subunits to poly(U). Note poly(U) appears near the top of the gradient, or accompanying the 28S subunit peak, when present, but none in faster sedimenting complexes. 28S subunits do not sediment in faster sedimenting complexes.

Table 7

Binding Analysis of Sucrose Density Gradients Poly(U)

Fig 18	28S Conc μM	28S SDG pmol	28S peak pmol	28S recov %	mRNA Conc μM	mRNA SDG pmol	mRNA recov %	mRNA 28S peak pmol	mRNA Not Bound pmol	mRNA Bound % of Total
A	0.0	0.0	----	----	0.18	18	37.6	----	6.76	----
B	0.36	36	17.1	47.5	0.0	0.0	----	----	----	----
C	0.36	36	12.6	35.0	0.18	18	30.7	4.46	1.02	80.7
D	0.72	72	17.6	24.4	0.18	18	33.6	6.05	0.83	87.9



B

5' ATTATGCTGAGTGATATCCTACAACAACAACAACAACAACAACAACA 3'

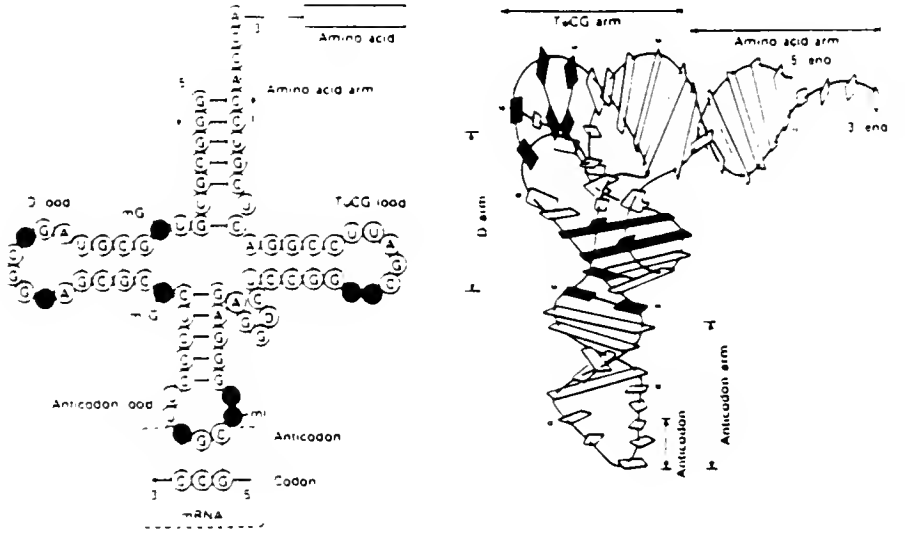


Figure 19. Non-Messages Used for Binding Assays. A: pGEM-3zf(-), Promega, linearized by HindIII before use; B: TO 139, ssDNA template; and C: tRNA, E. coli tRNA (Sigma). 5' end labelling described in Materials and Methods.

subunits, ribosomes were not saturated (i.e. no more than 2 pmoles of polynucleotide are bound). The small subunit's affinity for ssDNA was about 4 fold less than that of mRNAs, while the apparent affinity for dsDNA or tRNA was about 20- to 30-fold less than that for mRNAs (Table 8).

These results indicate that the 28S subunits have a relatively low affinity for non-message polynucleotides. It may be that the structure of the polynucleotides presented to the 28S subunits is incompatible with strong binding to the template binding site. The mRNA must offer the binding site something that the ss and dsDNA and tRNA could not.

Competition for 28S Ribosomal Subunit Binding of COII mRNA by Mitochondrial and Non-mitochondrial mRNAs

The binding of all tested mRNAs by the 28S subunits raised the question as to whether they all were bound at the same site or if they were bound at different locations on the same subunit. If ATPase 8/6 mRNA was interacting with the 12S rRNA (28S subunit) by a putative S-D sequence, then poly(U), poly(C), and COII mRNA would not compete for this site because they do not exhibit an S-D sequence.

Millipore filter assays were employed to determine if an unlabelled RNA could effectively compete for the binding of COII mRNA. Both mitochondrial and non-mitochondrial mRNAs were individually mixed with a saturating concentration (0.17μM) of radiolabelled COII mRNA (see figure 13) prior to incubation with 5 pmoles (0.1μM) 28S subunits.

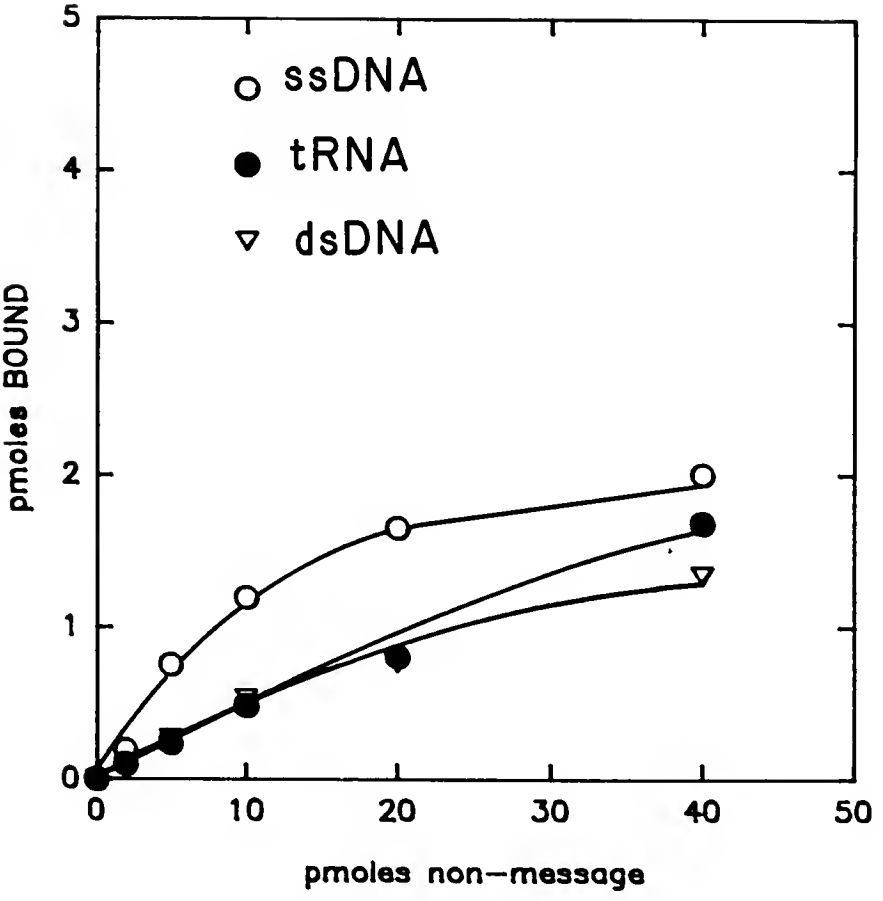


Figure 20. Binding of Non-message Polynucleoties by 28S Subunits. Labelled polynucleotides, 1-40 pmoles (0.02-0.80μM), were incubated in a 50μl final volume with 5 pmoles (0.10μM) of 28S subunits for 10 minutes at 35°C.

TABLE 8

Binding Analysis of Non-Message Polynucleotides

Polynucleic Acid	Affinity $K_d x10^{-9} M \pm sd$	Sites on 28S Subunits n±sd
pGEM (dsDNA)	811±98	0.55±0.12
tRNA	1600±96	0.96±0.06
TO 139 (ssDNA)	223±15	0.55±0.02

The competitors were increased in relation to COII mRNA from 0.5- to 4-fold molar amounts.

The ability of each of the RNAs tested to compete for the binding of COII mRNA with a close correlation to their apparent dissociation constants provides confidence that the RNAs are occupying the same site on the ribosome. The previous use of poly(U) as a translation template adds to the evidence that this binding is significant.

The data for these experiments were plotted as percent of control in Figure 21. The analysis was compiled using Enzfitter Kinetics software by Leatherbarrow (1987) and a personal computer. The modified equation for binding inhibition (Cantor and Schimmel, 1980) was used and is shown below:

$$Y= 1-\{ 1-r(I/K_i) \ / \ 1+r[1+I/K_i]\}x100$$

where $r= K_d/A$, constant=0.329, (K_d for COII mRNA is 56nM, Table 4, and A= concentration of COII mRNA, 0.17μM) and Y=1-vi/vo (vi/vo=percent inhibition).

Table 9 shows the results of the above data analysis. ATPase 8/6 and COII mRNAs and poly(U) were essentially equivalent competitors displaying a K_i around 50nM, which correlates well with the apparent K_d. Poly(C) competed less effectively for binding by the 28S subunit showing a 2 fold weaker inhibition of COII mRNA binding then the other three. The binding affinity for poly(C) is also 2- to 3-fold less than COII mRNA (Table 6), possibly for unusual structural characteristics. Again the apparent K_d and K_i correlate well.

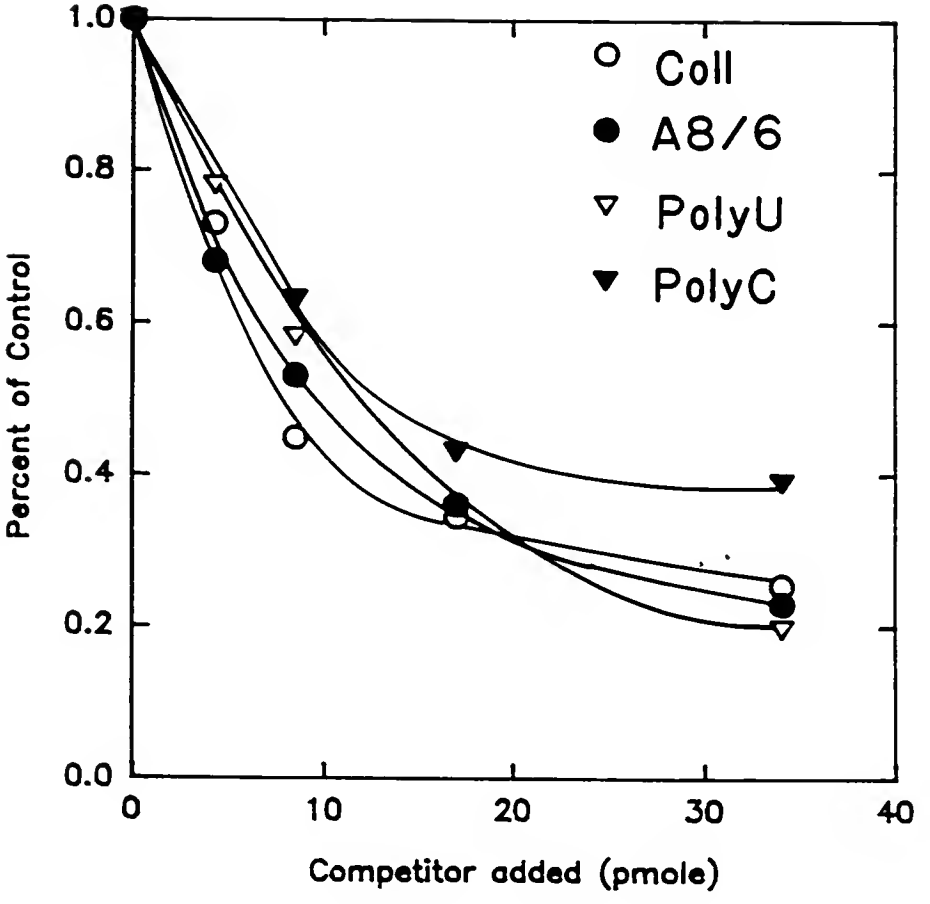


Figure 21. Competition by Other Templates for the Binding of COII mRNA on 28S Subunits. 28S subunits, 5 pmoles (0.1μM) were incubated with a mixture of 8.5 pmoles (0.17μM) of COII mRNA (100% of Control-saturating) and increasing concentrations of competitor from 4.3-34 pmoles (0.09-0.68μM) for 10 minutes at 35°C in a 50μl final volume.

TABLE 9

Analysis of Competition for COII mRNA Binding by mRNA Templates

Messenger RNA	K_i $x10^{-9}M$ $\pm$se
COII	51.5$\pm$3.9
ATPase 8/6	46.1$\pm$0.9
Poly(U)	47.8$\pm$4.7
Poly(C)	108$\pm$7.6

Effects of Aurintricarboxylic Acid on COII mRNA Binding to 28S Ribosomal Subunits

The 28S subunit is a complex of a 12S rRNA molecule and 33 proteins. This can be compared to the E. coli small subunit which possesses a 16S rRNA and 21 proteins. Speculation has frequently centered around the possibility of the proteins taking up functional as well as "space- filling" roles of the petite mitochondrial rRNA.

A computer aided search for possible complementary regions between the 12S rRNA and the mitochondrial messages has lead to speculation about the possibility of an internal S-D-like sequence on the mitochondrial messages (Saccone et al., 1985). Eight of the 13 mRNAs were identified as having a sequence complementary to a region of the 12S rRNA about 700 nucleotides from the rRNA 5' end. The sequence on the 12S rRNA is approximately 20 bases long and gives predicted energies of interaction (ΔG) between -20to -30 kcal/mole. This is 4-6 times the ΔG of -4.9 kcal/mole using Zuker values for strong S-D interactions (i.e., AGGAGGU of R17 phage protein A mRNA S-D sequence, all of which are paired). This kind of binding would appear to be too strong to allow enough freedom for the RNA to be translated without expending a great amount of energy.

The 12S rRNA is not accessible to modification by chemical agents in this region (Faunce,1991) giving evidence that a macromolecule like mRNA is not likely to penetrate this

domain either. Furthermore, the binding of COII mRNA was shown to have little effect on the modifications or cleavage of 12S rRNA (Faunce, 1991). The only effect noted was to two bases in a region approximately 500 bases 5' of the putative S-D sequence which expressed enhanced sensitivity to RNase A when COII message was bound. Another possibility for rRNA to be involved in the binding interactions with the mRNA would invoke a magnesium cross-bridging model that would be novel in translation systems.

A final consideration would be to picture this interaction like any other RNA binding protein-RNA interaction. Binding of mRNA by the mitochondrial ribosomal proteins seems the most likely to occur, since all mRNAs, regardless of sequence and structure, were able to be bound most likely in the same site with approximately the same affinity by the 28S subunit. In this case a red dye, Aurintricarboxylic Acid (ATA)(Figure 22) was used as an inhibitor of protein-RNA interaction by the ribosome. This dye has been used successfully as an RNase inhibitor and its interaction on RNA binding proteins has been characterized (Gonzalez et al., 1980). It was shown to inhibit protein chain initiation of eukaryotic and prokaryotic ribosomes but not the continuation of peptidyl elongation at low concentrations (5μM)(Mathews, 1971).

To test the effects of ATA on the binding of mRNA by 28S subunits, 5 pmoles (0.1μM) 28S subunits were preincubated for

5 minutes at 35°C with 5μM ATA. This concentration of ATA is strongly inhibitory to RNA binding proteins (Blumenthal and Landers, 1973 and Marcus, et al., 1970). It also is capable of inhibiting the binding of COII mRNA from 0.17-0.68μM (saturating levels) by the 28S subunit suggesting that the message is being bound by proteins on the small subunit which serve as the mRNA binding site. The data were plotted in Figure 23. As can be readily seen, the virtually complete ablation of the 28S subunits ability to bind COII mRNA was observed in the presence of ATA.

Conclusions

The mitochondrial ribosomal small subunit was able to bind not only mitochondrial mRNAs but non-mitochondrial mRNAs, including homopolymers of uridine and cytosine not expected to exist in nature. That the 28S subunit showed no preference for homologous messages, registering dissociation constants around 40-50nM with the exception of poly(C) (>0.1μM), implies that all of these test templates have properties that allow them to be bound by the mitochondrial small subunit. Since poly(U) binds with the same affinity as the mitochondrial messages, it appears that special secondary structures of these messages are not a requirement for their binding by 28S subunits. The site to which these templates are binding then appears to be a general template binding domain, whose

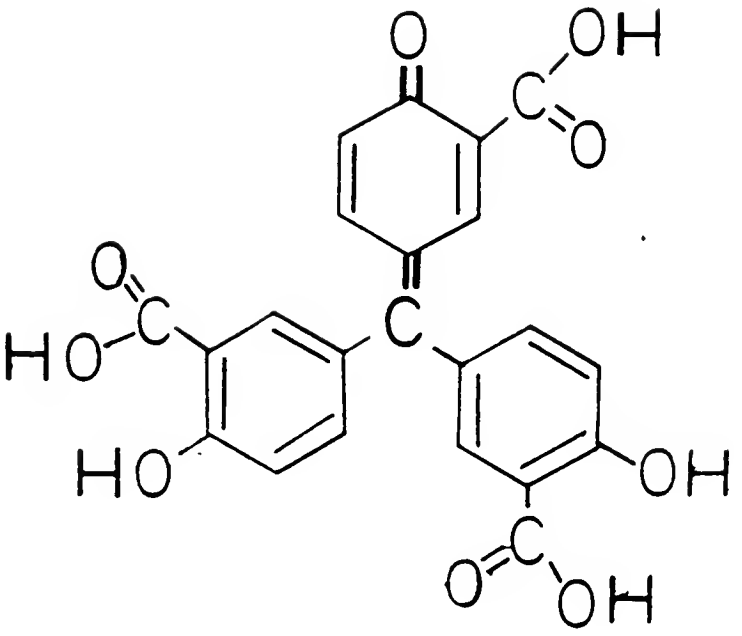


Figure 22. Chemical Structure of Aurin Tricarboxylic Acid.

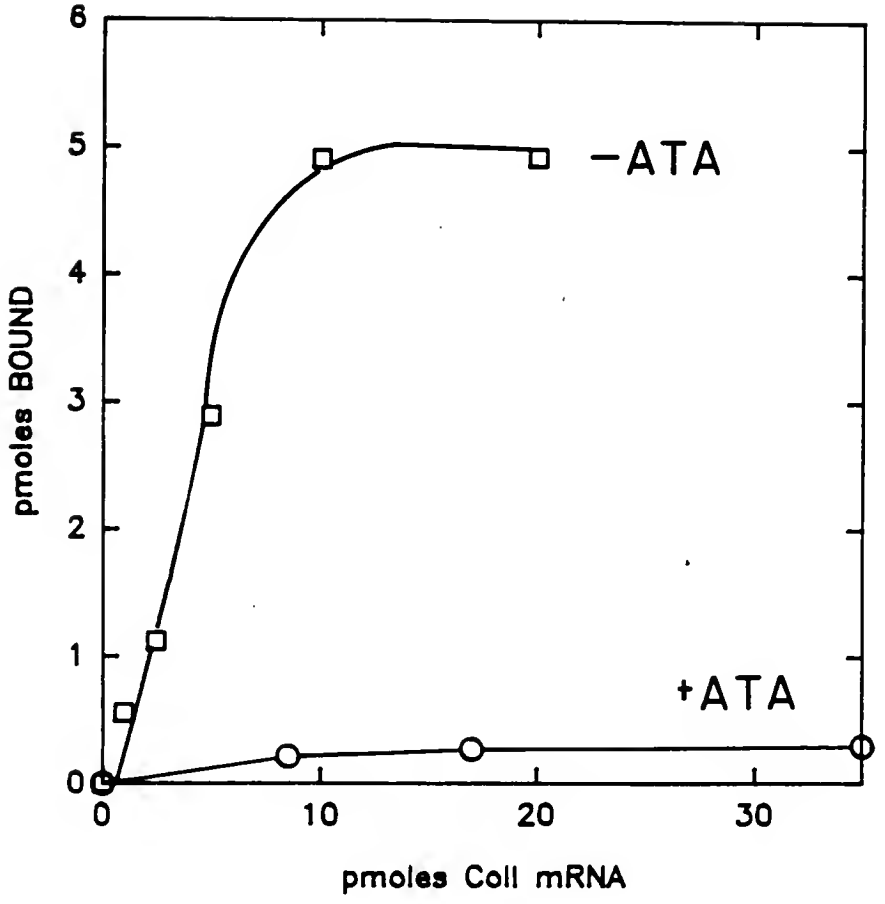


Figure 23. Effect of ATA on CoII mRNA Binding to Ribosomes. 28S subunits, 5 pmoles (0.1μM) were incubated with 5μM ATA prior to the addition of 8.5-34 pmoles (0.17-0.68μM) radiolabelled CoII mRNA. Note that 0.17μM is a saturating concentration of CoII mRNA for 0.1μM ribosomes.

properties were inferred earlier (Denslow et al., 1989). Perhaps the common feature of these different templates that allows binding is the presence of (relatively) unstructured, single stranded regions (since poly(U) binds with the same affinity as COII and ATPase 8/6 mRNA, and competes for the same binding site). Furthermore, since COII and ATPase 8/6 mRNAs bind with the same affinity and compete for the same binding site, it would appear that the putative S-D interaction of ATPase 8/6 is not contributing significantly, if at all, to the binding of this mRNA to the ribosome.

The mitochondrion's compartmentalization, alone, is a limiting agent which would allow only the presence of its own mRNAs for translation by the ribosome. The fairly high affinity measurements, the apparent single site, and reversible binding, and the ability of several mRNA templates to be bound point to this binding as a physiologically meaningful interaction. The sucrose density gradients exhibited the small subunits high affinity for COII mRNA and poly(U) and the unit stoichiometry of binding. It is doubtful that the mitochondrion could support much in the way of polysomes because of the relative paucity of ribosomes and mRNAs. Each liver cell has an average of $4\text{-}5 \times 10^5$ mitochondrial ribosomes (O'Brien, personal communication). The number of messages per adult rat liver cell mitochondrion has been determined at about 90 messages per mitochondria or 8 molecules of each message. The 12S rRNA content is 81 per

mitochondria (Cantatore et al., 1984) so that the ratio of message to 12S rRNA (the maximum number of small subunits possible) is about 1:1. While this does not preclude the formation of polysomes in vivo, it does show that the stoichiometry does not favor them. In HeLa cells each of the 11 messages are produced at a rate of roughly 1 molecule per minute and demonstrated half-lives between 25 and 90 minutes and the 12S rRNA is about 100 fold higher (Gelfand and Attardi, 1981), all in a protein dense matrix where mobility would be severely limited. Thus, it stands to reason that it may be advantageous for the small subunit to sequester a message with a fairly strong affinity interaction until the balance of the translation machinery components arrive to initiate the peptide elongation.

Furthermore, the binding appears to be predominantly to a proteinaceous site. Previous work with RNA modifiers DMS and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimde metho-p-toluenesulfonate (CMCT) followed by AMV-RT extension has been done on 12S rRNA in the presence and absence of COII mRNA. These chemical agents are capable of modifying solution accessible adenines and cytidines (DMS) and guanines and uridines (CMCT) on the N1 nitrogen of the purines and the N3 nitrogen of the pyrimidines with which they react (see figure 4). These nitrogen molecules are involved in Watson-Crick base pairing interactions and will not be modified by the chemicals if they are paired. The experiments showed that the

12S rRNA chemical modifications were not different with or without bound COII mRNA. The putative S-D-like sequence was also shown to be unavailable for modification in the 28S subunit by any method, chemical (DMS and CMCT) or enzymatic (RNase T_1, A, and V_1), and was also predicted by phylogenetic analysis (Gutell et al., 1985) and by computer folding analyses to be involved in base-paired helices. These data would indicate that the 12S rRNA sequence that has regions of complementarity in the eight mitochondrial messages is unavailable for interaction with the mRNA.

Robert Heck has bound a bromouracil-containing (a UV activated uridine analog), radiolabelled COII mRNA to 28S subunits. The bound message is then UV cross-linked to proximal elements of the binding site of the small subunit. After degrading the mRNA and dissembling the subunit, the crosslinked label (mRNA fragment) was found to be associated with two ribosomal proteins with molecular weights in the 40 kd range.

The binding, then, is consistent with the speculation of a "generic mRNA binding site" on the 28S subunit. What, then, are the properties required for binding to this site? First, ssRNA binds well, and DNA (ds or ss) or dsRNA bind poorly, suggesting that the $C_{3'}$-endo "Pucker" characteristic of polyribonucleotides may be a significant feature required for ribosome binding, as well as, an open stretch of "unstructured" RNA. Because the affinity of poly(U) for the

binding site matches that of COII, both may be binding by the same kind of interaction, involving predominantly unstructured, single stranded regions of the template. This possibility raises questions: What is the structure of COII mRNA? Is it largely single stranded, or are only one or a few regions single stranded/do one or more of these get bound by the 28S subunit binding site? Where are they located? Finally, does the binding of COII message to the small subunits include the 5' end and therefore the initiation codon?

ANALYSIS OF THE SECONDARY STRUCTURE OF BOVINE MITOCHONDRIAL COII MESSENGER RNA

Background and Significance of Secondary Structure Analysis

Some insights into the nature of the mRNA at the site of interaction with the 28S subunit were revealed in the previous studies dealing with affinity, specificity, stoichiometry, reversibility, and the nature of the binding site (RNA or protein). Access to a computer prediction of the secondary structure of COII mRNA may provide some clues into physical nature of the molecule and some understanding of the potential site of interaction with the small subunit.

Computer nucleic acid folding programs predict the secondary structure of a polynucleotide based on conventional and nonconventional but common Watson-Crick base pairing (i.e., the potential for guanines to pair with uridines. Most folding programs are designed to minimize the energy of the final folded product (maximize the number of the most favorable base pairing interactions) and/or to meet phylogenetic restrictions. Neither of these may provide a complete or accurate description of the mRNA structure, but they do give a projection on which to build a more accurate model.

To obtain data useful for modelling the mRNA structure, the RNA must be physically examined in a manner that does not disrupt its higher order structural characteristics. These data can be used to refine the computer prediction and deliver a comprehensive view of the physical nature of the message. The techniques frequently used to study the structure of macromolecules: X-ray crystallography and electron microscopy, requires a great deal of precious reagent and/or use fixatives and procedures that may be destabilizing to base pairing. Furthermore, they provide only limited information on molecules like bare RNAs.

An alternative method used to differentiate the stems and single stranded stretches of an RNA, and the one chosen here, is the chemical modification and enzymatic cleavage of the RNA followed by primer extension termination (Moazed and Noller, 1986; Stern et al., 1988). This technique captures the target RNA's bases in their higher order structure and AMV-RT driven primer extension discloses the modified base as the extension is disrupted (Boorstein and Craig, 1989; Knapp, 1989).

The use of chemical probing modifies both open and potentially secluded single stranded nucleotides areas. Dimethyl sulfate (DMS) methylates adenines (N1) and cytidines (N3)(see Figure 4) that are not paired with another nucleotide forming a stem or some other structure, like a pseudoknot. Guanines are also a target for the action of this modifier but are generally not disclosed by this method in that they do not

halt AMV-RT polymerization (Guanines may be disclosed by AMV-RT extension if the RNA is chemically cleaved by aniline treatment before extending).

The use of enzymes to probe the RNA opens the molecule to a different sort of physical investigation. The enzymes used in this study, ribonucleases A and T_1, recognize the bases of the polynucleotide but cleave the phosphodiester "backbone" of the RNA. This leaves a 3' phosphate on the 5' segment of the molecule. RNase T_1 cleaves the RNA at single stranded guanines and RNase A cleaves the RNA at single stranded cytidines and uridines. Another nuclease, cobra venom endonuclease, identifies structured portions of the molecule and cleaves the phosphodiester backbone to leave a 5'-phosphate. This structure need not be a double stranded helix in the RNA, simply a helical structure (Lowman and Draper, 1986).

The disclosure of the modification event is accomplished by primer extending a copy DNA (cDNA) of the modified message. The technique employs [α-^{32}P] dATP and AMV-RT with primers complementary to specific regions in the RNA to produce labelled cDNAs. These cDNAs will terminate at the site of modification, cleavage by nucleases or methylation by DMS. They are then run on denaturing gels and observed by autoradiography. The actual band marking the site of modification will always appear one base further down in the gel then the affected base.

Computer Modelling of the Secondary Structure of COII mRNA

The first folding program used in this study was an early Zuker program called FOLD. This program was easy to use and conveniently located on the ICBR VAX (Digital) as part of the Genetics Computer Group (GCG) package (Zuker and Stiegler, 1981). The program relies on thermodynamic parameters of base pairing interactions, stem length and composition and loop size along with computer algorithms to establish minimum energy foldings. Like other fold programs, FOLD was tested on well studied RNAs like specific tRNAs, the structures of which have been studied by X-ray diffraction of crystallized tRNA, enzymatically and chemically probed, and phylogenetically predicted.

The primary advantage of energy minimization over a phylogenetic program is that only one sequence is required. Phylogenetic programs predict secondary structure by aligning several sequences that are known to have similar function followed by folding them into analogous structures. The cardinal algorithm for the phylogenetic prediction is the identification of compensatory base changes that maintain complementarity, through the analysis of covariant bases in the molecule. The value of this approach has been proven in predicting rRNAs secondary structure where folding programs have failed.

The sequence for COII mRNA was analyzed using the FOLD program, and the first structural prediction was drawn using Squiggles, an output program for FOLD (Figure 24). The extensive structure predicted by this program lead to speculation into the manner of how the folding of the message might occur *in vivo* and how to simulate it.

An improvement to the FOLD program would be an algorithm that gave preference to locally formed stems over long-distance interactions. This result could be engendered by folding the message twenty bases at a time and resolving which structures would form based not only on thermodynamic considerations but also on preference for locally formed structures. This labor intensive and time consuming process was called a "Walk-Through" and it was still incapable of accepting physical data input.

At about the time the walk-through was being completed a new program became available, LRNA (Jaeger, *et al.*, 1990). The LRNA algorithm incorporated the preference for locally formed structures, as well as, other data about the stability of various stem/loop conformations, like "tetra-loops" (Woese, *et al.*, 1990), would allow for "suboptimal" folds and the data from physical probing, by far the two most important advancements of LRNA. In order to *show* the usefulness of this program further detail will be discussed under the *Refinement of the Secondary Structure of COII mRNA* subheading.

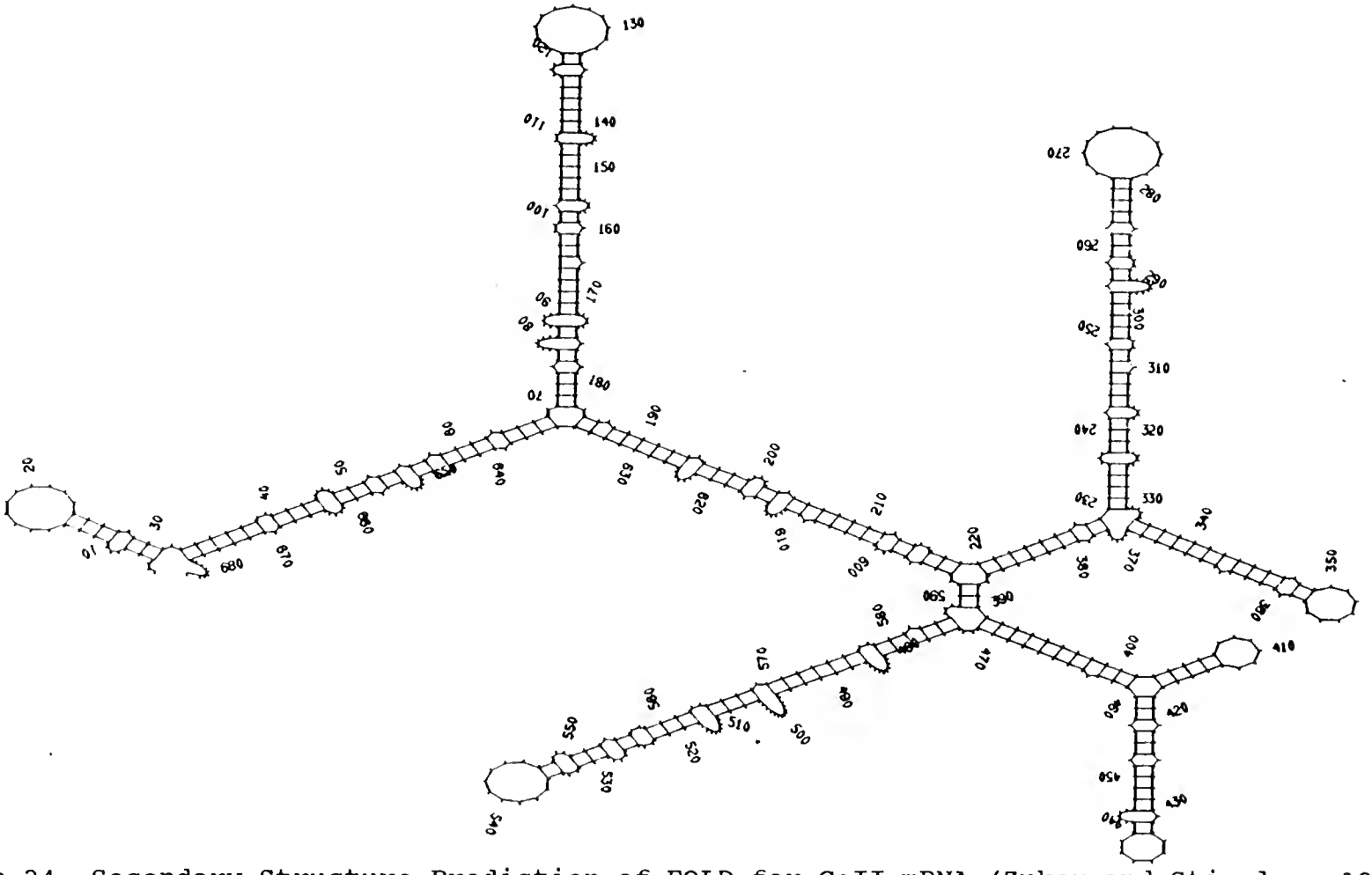


Figure 24. Secondary Structure Prediction of FOLD for CoII mRNA (Zuker and Stiegler, 1981). The output is done by Squiggles a drawing program of GCG. Note the extensive structure predicted by energy minimization alone.

Enzymatic Probing of the Secondary Structure of COII mRNA

Enzymatic probing of the COII mRNA was expected to provide data on the highly accessible regions in the structure. Single stranded uridines, cytidines, and guanines were probed with RNases A and T_1. Potentially double stranded segments were probed with V_1 endonuclease.

After modification, primers specific to the RNA were annealed and extended (see Materials and Methods, Extension of Oligodeoxyribonucleotide Primers). AMV-RT extension will be halted where the modifier has acted and this site will appear as a band on a denaturing gel at the position just 3' of the site of modification. Proper titration of the modifier allows for a single modification every 150 bases of the molecule (see Figure 10). This is roughly the limit of resolution on the 6% denaturing gels used and about the distance between each of the five primers.

Examples of the kind of data that were compiled for RNase A and T_1 are displayed in Figure 25 for extension Ω and Figure 26 for extension β. Cleavages by RNase A can be seen at C6, U8, U21, and C29 and with RNase T_1 the G23 and 24. Further 3' in the molecule, using the same primer, RNase A is seen to cut at U60 and C65, 71, 77, and 79. RNase T_1 has an effect visible on G92. On the β extensions in Figure 26 RNase A activity is apparent on U291, 300, 311, 314 and C288, 298, 308 (weakly) while RNase T_1 shows G312, 335, 351, and 356.

Cobra venom endonuclease, V_1, provided very few data points, as is typical for this enzyme. Examples of V_1 modifications are shown in Figure 27 for Ω and α extensions.

All enzymatic data (experiments were done in triplicate and usually repeated at least once) were compiled and loaded onto a primary sequence structure (Figure 28) acquired from GENBANK database through the GCG package on the ICBR VAX (Digital). One base, position 201, was a common variant of cytosine for uridine. Obviously these data alone is insufficient to determine the secondary structure, but it does provide some clues into readily accessible nucleotides on the message and their lack of involvement (for RNase A and T_1) or involvement (for V_1) in structures. This demanded more data before proceeding with the secondary structure modelling.

Chemical Probing of the Secondary Structure of COII mRNA

The use of DMS to probe the COII mRNA structure was expected to provide information about all target nucleotides accessible from solution. DMS is also a very reactive molecule so that its modification of target bases will be inhibited only by base pairing and its limited half-life in aqueous solutions, about two minutes. Cytosines are less reactive with DMS so methylation on these molecules is not as frequent as that of adenines (McSwiggen, 1991).

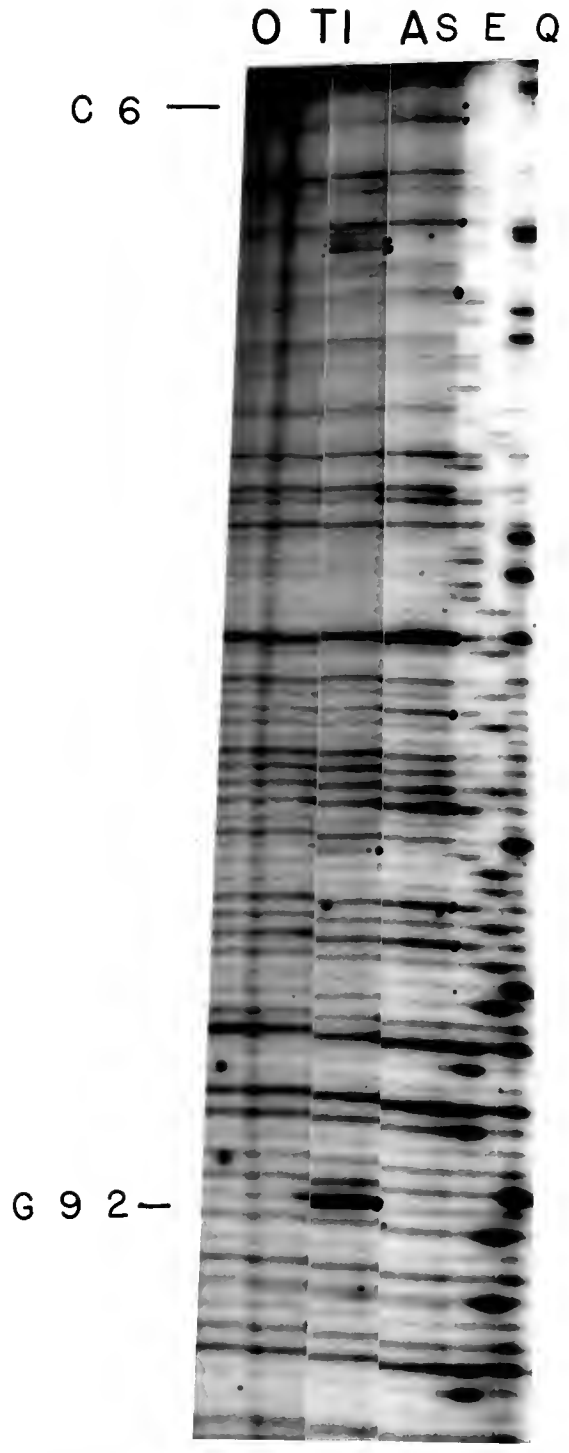


Figure 25. RNase A and T_1 Modifications on Ω Extensions. 0: control- no nuclease; A: RNase A modified; T_1: RNase T_1 modified; and SEQ: A,C,G sequencing ladder. Dots- • indicate modifications by nucleases.

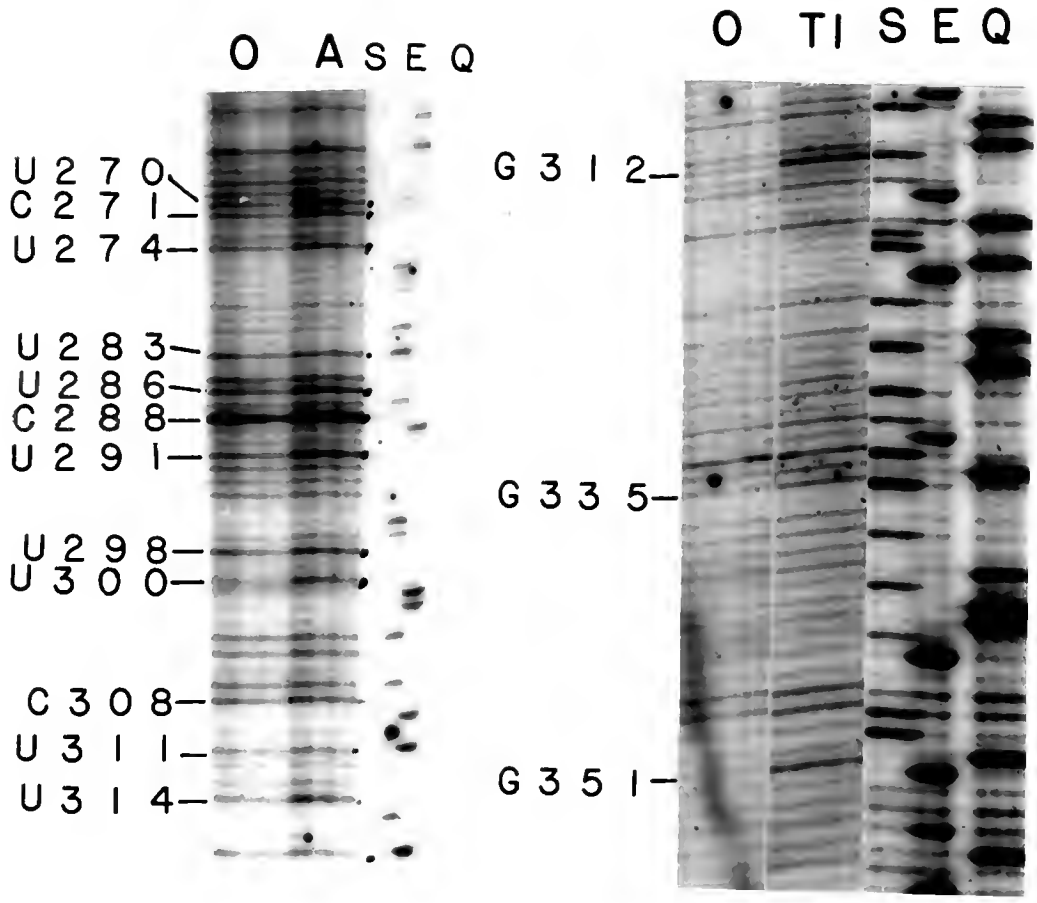


Figure 26. RNase A and T_1 Modifications on β Extensions. 0: control- no nuclese; A: RNase A modified; T_1: RNase T_1 modified; and SEQ: A,C,G sequencing ladder.

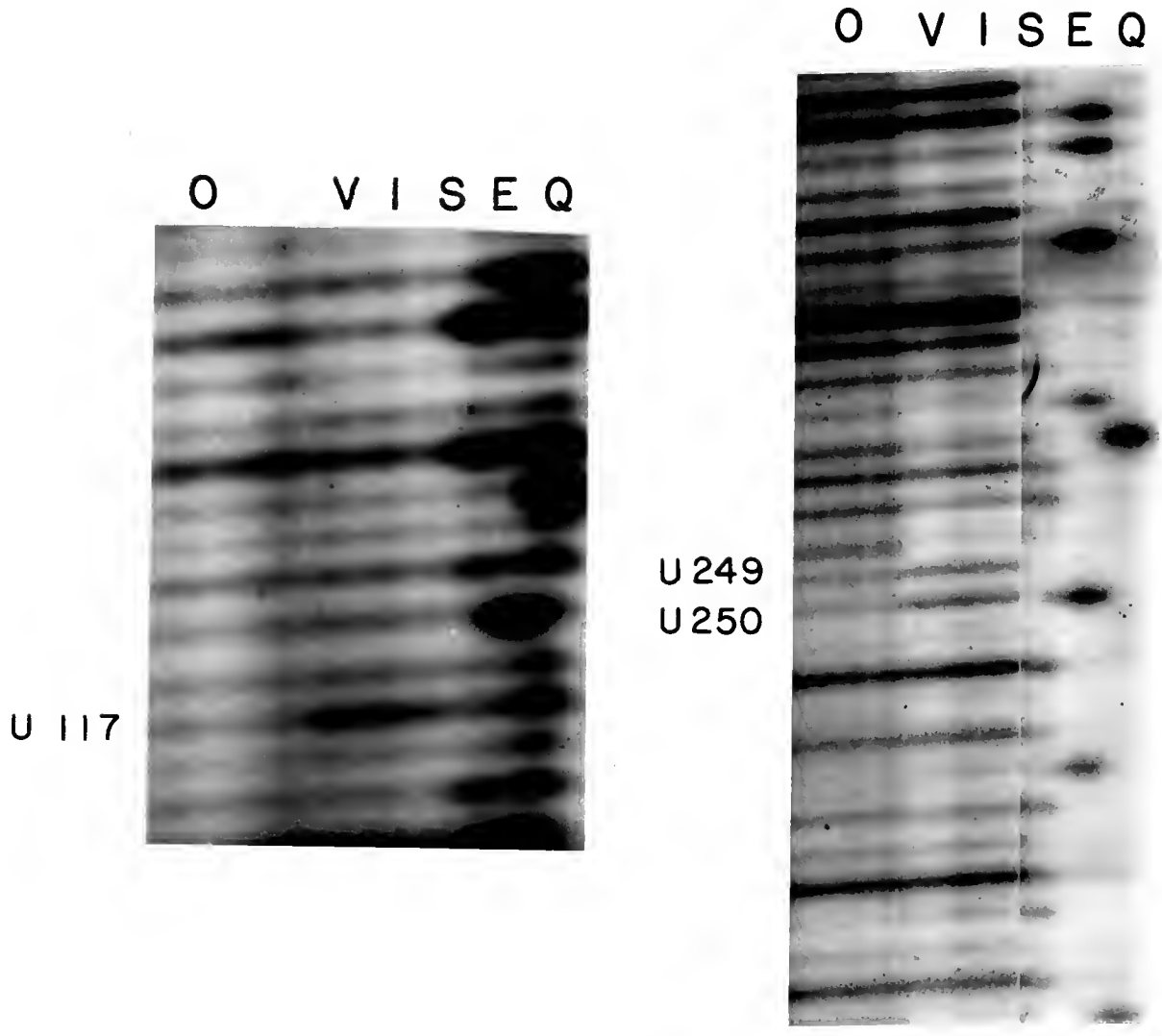


Figure 27. V_1 Modifications on Ω and α Extensions. 0: control-no nuclease; V_1: V_1 endonuclease modified; and SEQ: A,C,G sequencing ladder.

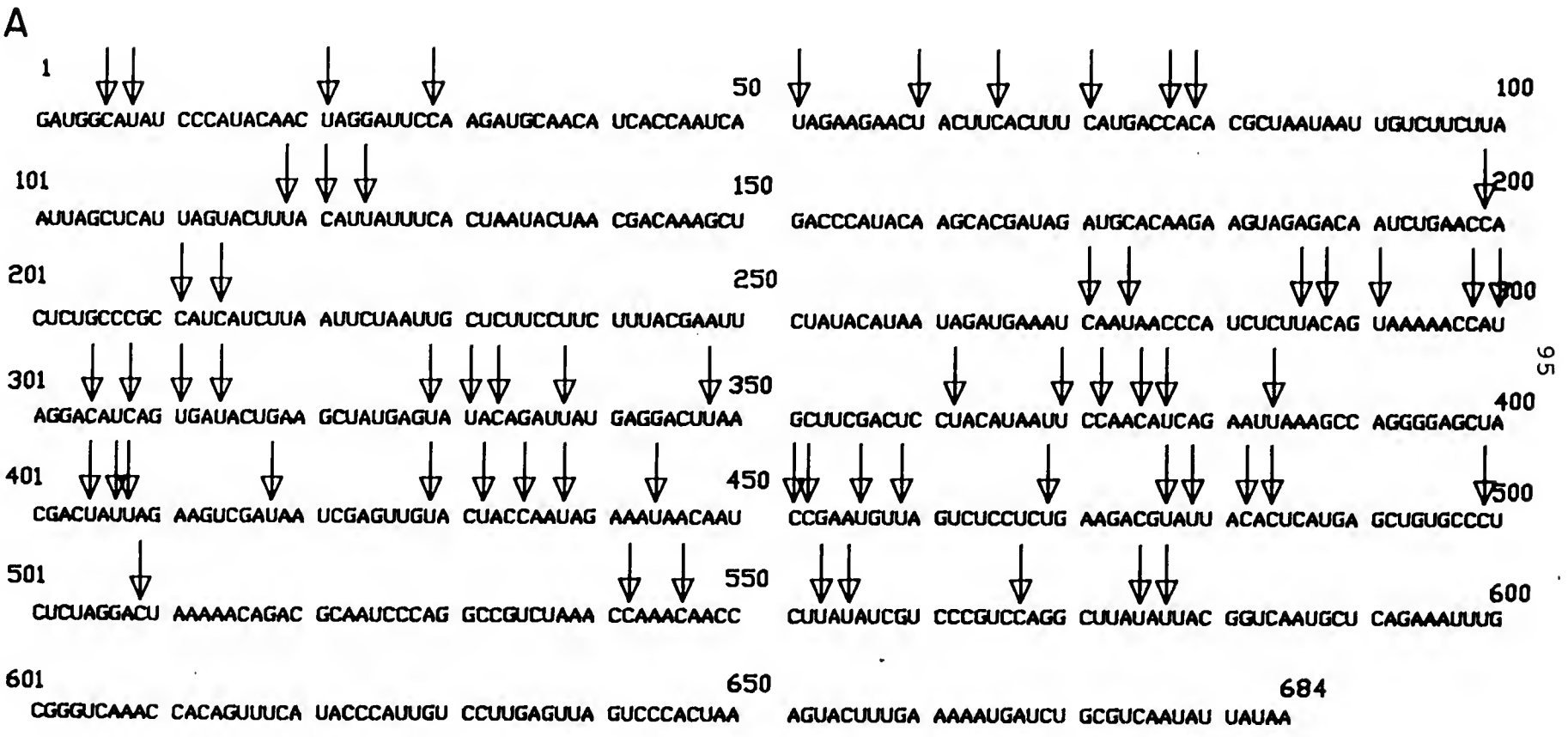


Figure 28. Nuclease Modifications on CoII Sequence. A) RNase A modifications, B) RNase T_1 modifications, and C) V_1 modifications. (Arrows indicate sites of nuclease modifications).

B

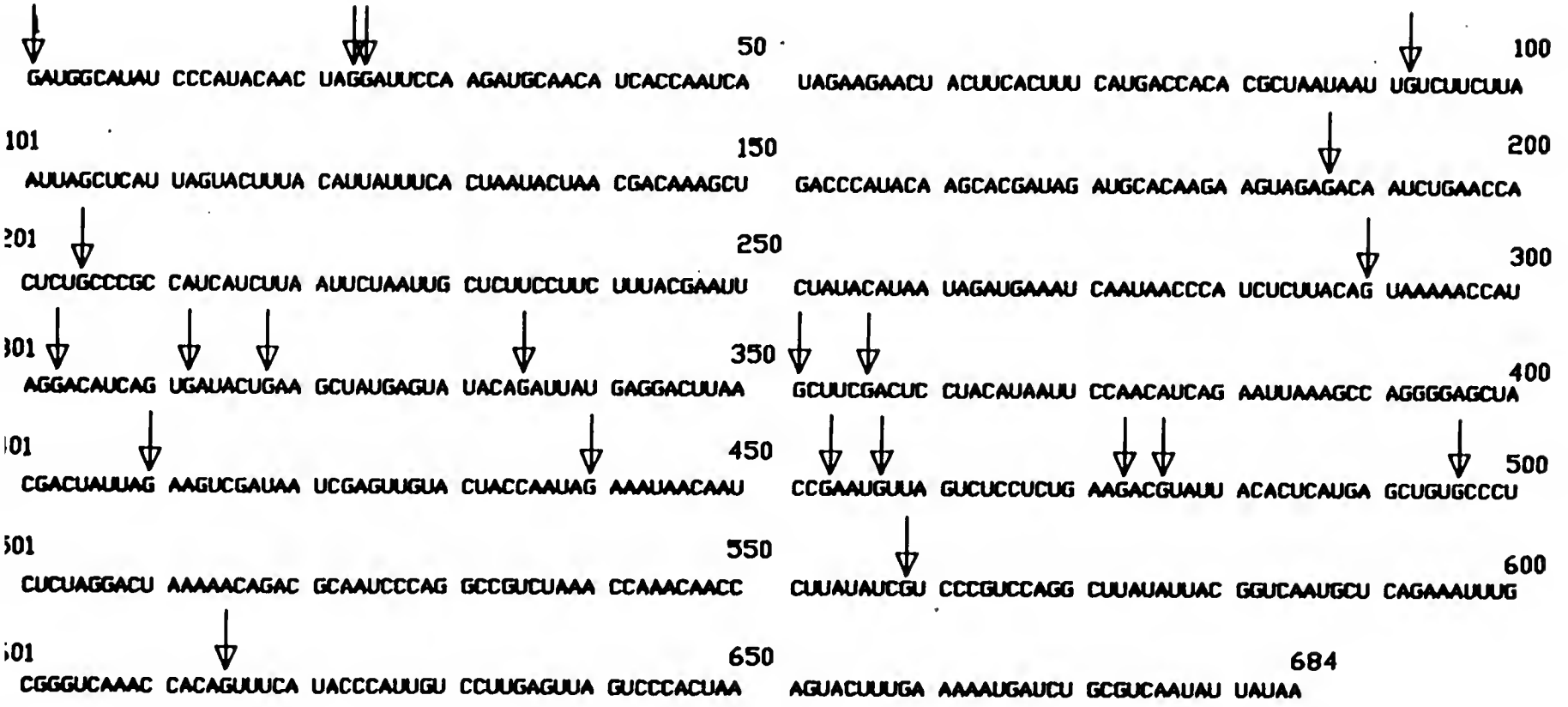


Figure 28 (cont.)

C

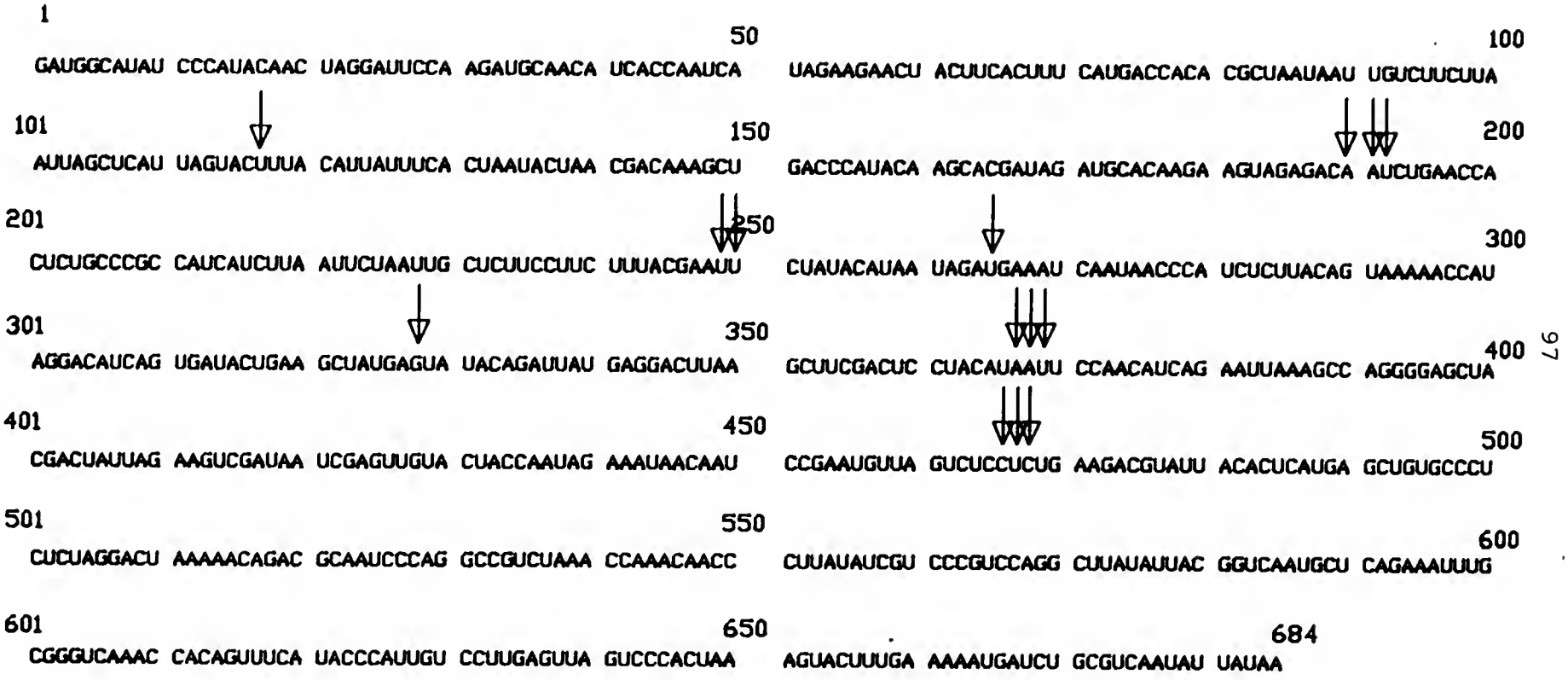


Figure 28 (cont.)

Examples of DMS modifications are displayed in Figures 29 and 30, for extensions Ω and β, respectively. Bases that are modified can be seen at A 46, 47, 52, 57, 58, 61, 66, 88, and 89 etc. and C59 and 81 weakly (DMS is less reactive on cytidines) in figure 29. The β extensions reveal a number of methylated adenines in a stretch between 292 and 340, as indicated on figure 30.

The data (experiments in triplicate and typically repeated at least once) were again compiled and loaded onto a primary sequence structure to see the areas accessible to solution on the message versus open regions accessible to nucleases (Figure 31). Note that regions accessible to DMS are not always accompanied with modifications from nucleases, (e.g., 30-70 and 220-245). These nucleotides may arguably be in areas sequestered from nucleases. Bases accessible to both V_1 and DMS, like A190 and 191, may indicate helical stretches of single stranded nucleotides.

There was a considerable amount of difficulty accumulating data on the 3' end of the molecule. The loss of data on the 3' end of the molecule may be attributable to the modification process itself or losses of material subsequent to modification, but prior to primer extension because sequencing extensions (no modifications) proceeded as expected.

DMS data collection may have been complicated (modifications reported out to base C625, 60 bases from the

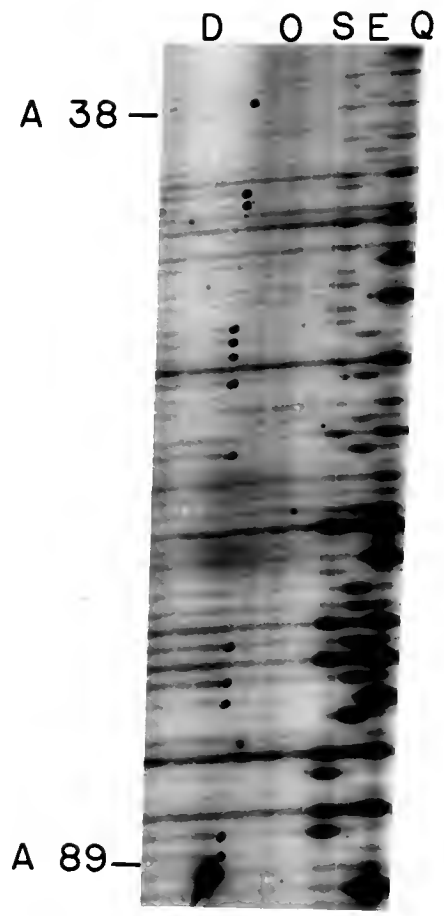


Figure 29. DMS Modifications on Ω Extensions. 0: Control- no DMS; D: DMS; and SEQ: A, C, and G sequence ladder. Dots- indicate modifications by DMS.

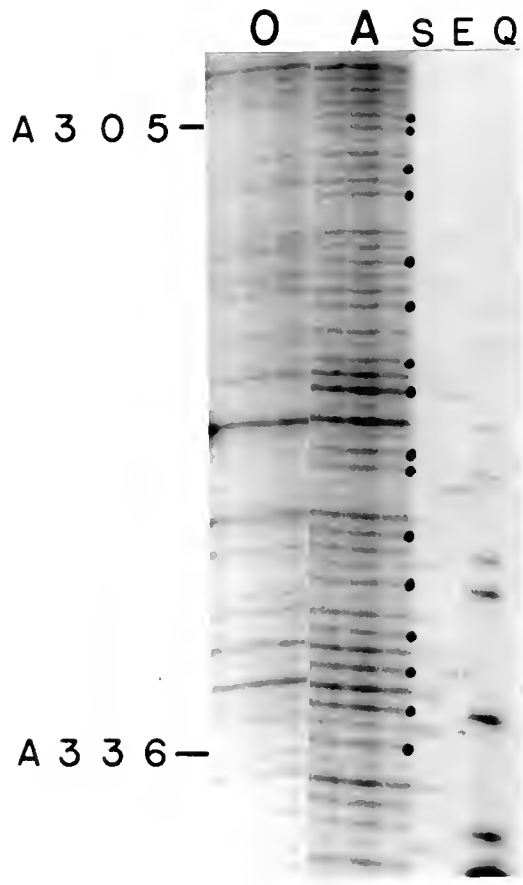


Figure 30. DMS Modifications on β Extensions. 0: Control- no DMS; D: DMS; and SEQ: A, C, and G sequence ladder. Dots- indicate modifications by DMS.

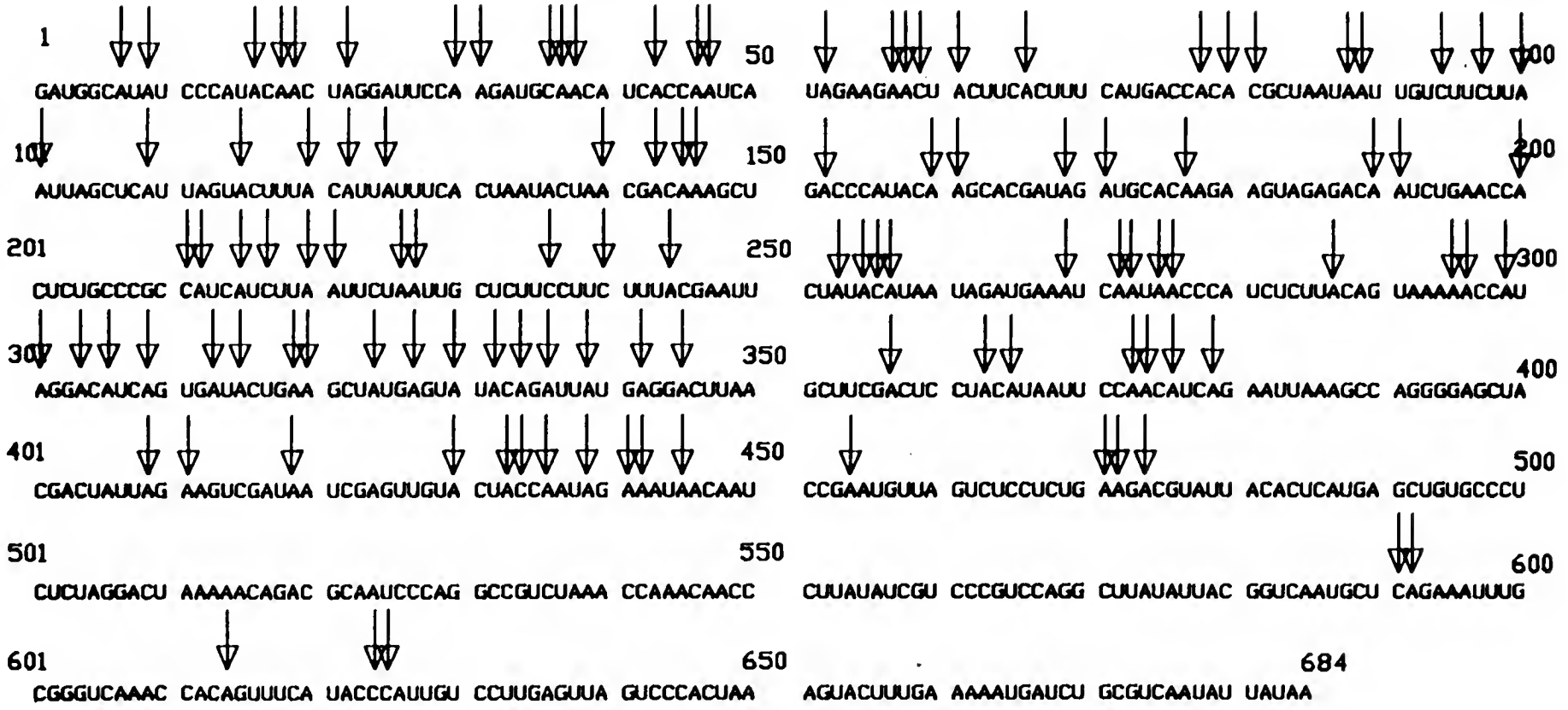


Figure 31. DMS Modifications on CoII Sequence. (Arrows indicate DMS modifications).

3' end) due to the adenine rich final 25 bases of COII mRNA, six of which are located immediately 3' of the primer. This region if it is single stranded and, therefore, modifiable may have caused a problem with annealing and/or early termination. Bases methylated by DMS are no longer capable of base pairing interactions and would inhibit the annealing of the primer.

The nucleases did not yield data until approximately 70 bases into the message from the 3' end. This can be explained by the very short segments of message resulting from nuclease cleavage of the 3' region. During the purification steps the modified RNA is subjected to several extractions, ethanol precipitation, and a subsequent wash. While the modified message is accompanied by carrier tRNA, disproportionate losses of the small polynucleotides are still inevitable. The resumption of modifications farther into the molecule (G615) and the unaffected sequencing extensions lend credence to this explanation.

An alternate explanation for the lack of reports by the single stranded modifiers in this region is that the 3' end of the molecule is not like the prediction of LRNA, but rather extensively structured. V_1 cleavages were not noted in this region either which seems to contradict this explanation. However, the use of titrations of the various nucleases and DMS should in any of these possibilities render a satisfactory prediction of the 3' end.

Refinement of the Secondary Structure of COII mRNA

The modification data were then compared to the structural prediction of the FOLD program (Figure 31) to see if the prediction was satisfactory. Plainly the FOLD program fails in its attempt to accurately predict the secondary structure of COII mRNA and the modifications reveal that the structure cannot be nearly so extensive. So the program for LRNA was requested from Douglas Turner and loaded onto the ICBR VAX (Digital). Coupled with its ability to assimilate the chemical and enzymatic data compiled and using the suboptimal folds algorithm it was hoped that LRNA could predict more probable secondary structures that are consistent with the chemical and enzymatic modification data.

The drawbacks to this program are computational time and storage space for the output files required. About 27 hours of main frame computer (CPU) time were required for folding the 684 base COII mRNA, estimated at $(5.2 \times 10^{-8}) \times N^{3.7}$ minutes, where N=the number of bases to be folded. An additional 5000 blocks were added to our VAX account to accommodate the save files, which required 3×10^6 bytes ($6.1 \times N^2$) of storage per optimal fold (Jaeger *et al.*, 1990).

The primary sequence was entered using an altered lettering sequence used by the program to identify bases that were modified. The program was run in BATCH, submitted during evening hours. The suboptimal plots provided a number of

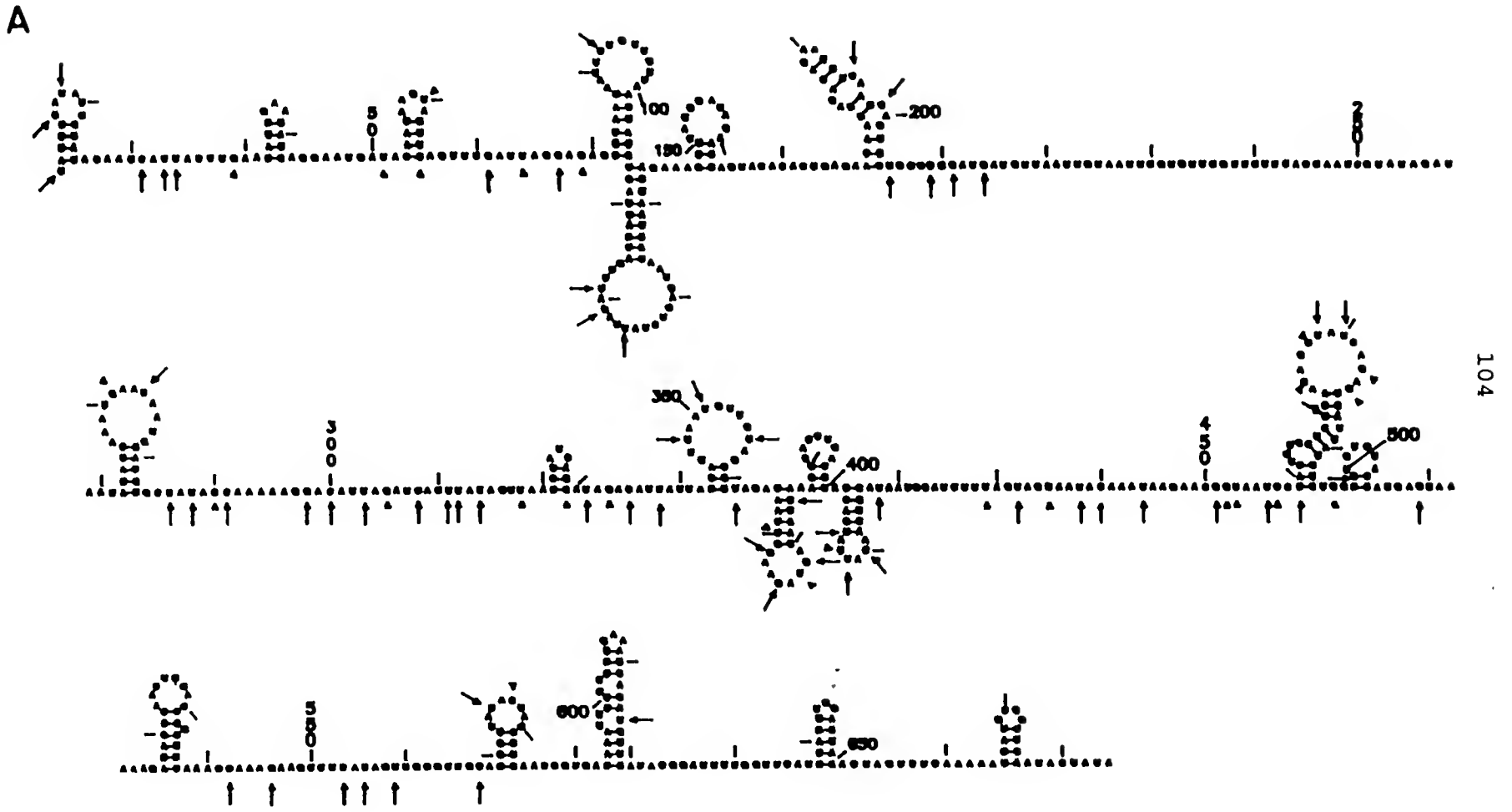


Figure 32. Model for the Secondary Structure of CoII mRNA. A) RNase A and T_1 modifications only; B) DMS modifications only; and C) V_1 modifications only. (Arrows indicate sites of modification). See figures 28 A-C and 31 for the base modified.

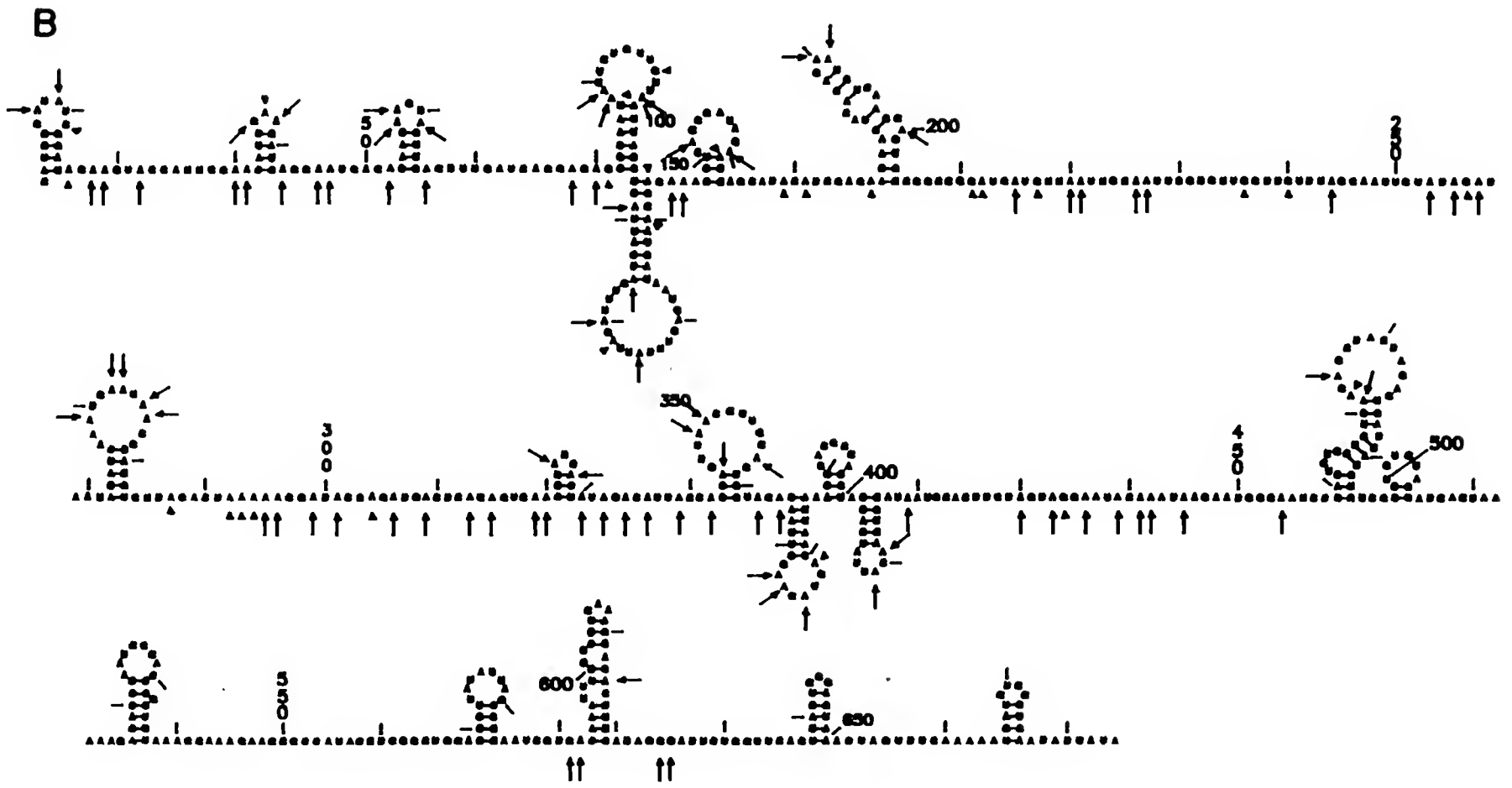


Figure 32. (cont)

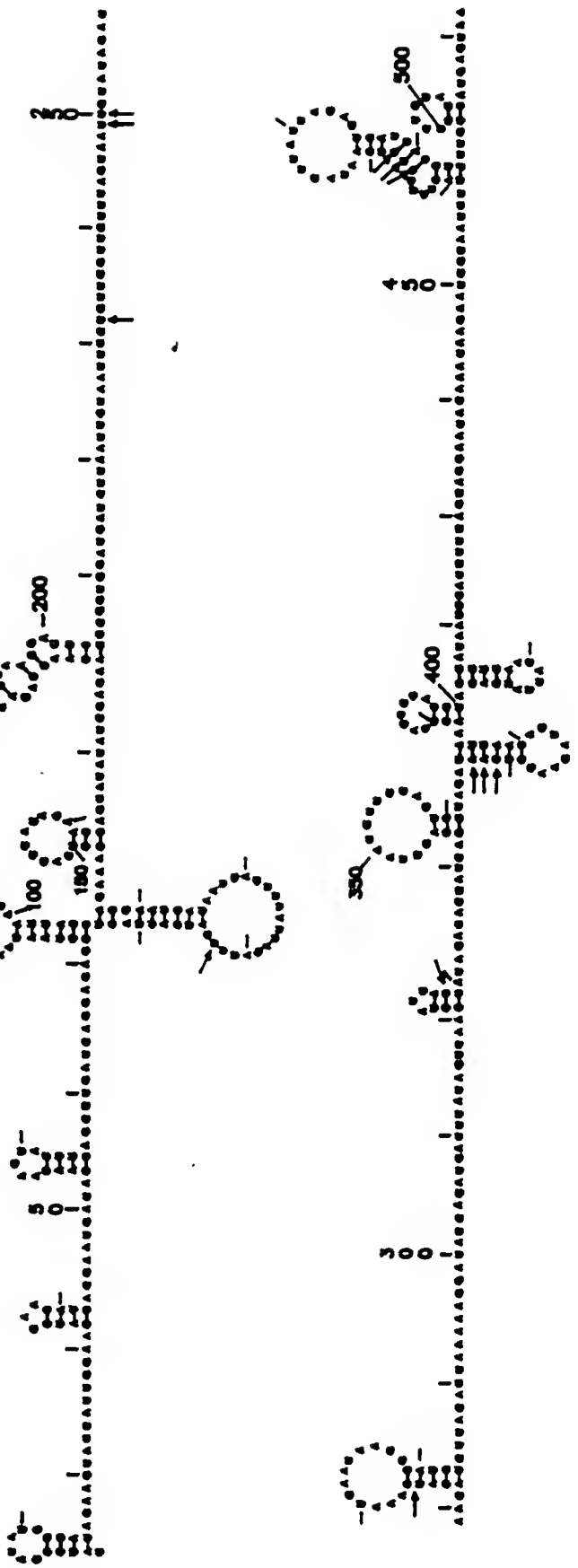


Figure 32. (cont)

alternative structures from which to choose. Here the bias of locally and first formed 5' to 3' structures were used for the final model. LRNA was not capable of using the Squiggles program available and the drawings were not particularly manageable and/or compatible for further manipulations anyway.

The structure was drawn on a Gateway 2000 386 personal computer with the use of a Logitech mouse and AUTOCAD 10.0, a computer-aided design software package. The model in Figure 32 is the final predicted secondary structure of COII mRNA based on physical probing and the predictions of the LRNA program.

Indications of Additional Structures on COII mRNA

The remainder of nucleotides not predicted (LRNA) to be in helical stems and not found to be modified by DMS or the enzymes used, requires further examination. It may be that these bases are simply tucked away in pockets in the tertiary structure of the mRNA, inaccessible to any of the modifiers. Or they may be in structures not predicted by the LRNA folding program. Little can be done to elucidate the micro-environments required to satisfy the first possibility. The second, however, may be checked using the WORDSEARCH program available in the GCG package on the ICBR VAX (Digital).

In order to disclose potential long-distance base pairing interactions, all stretches of 4 or more bases were put into

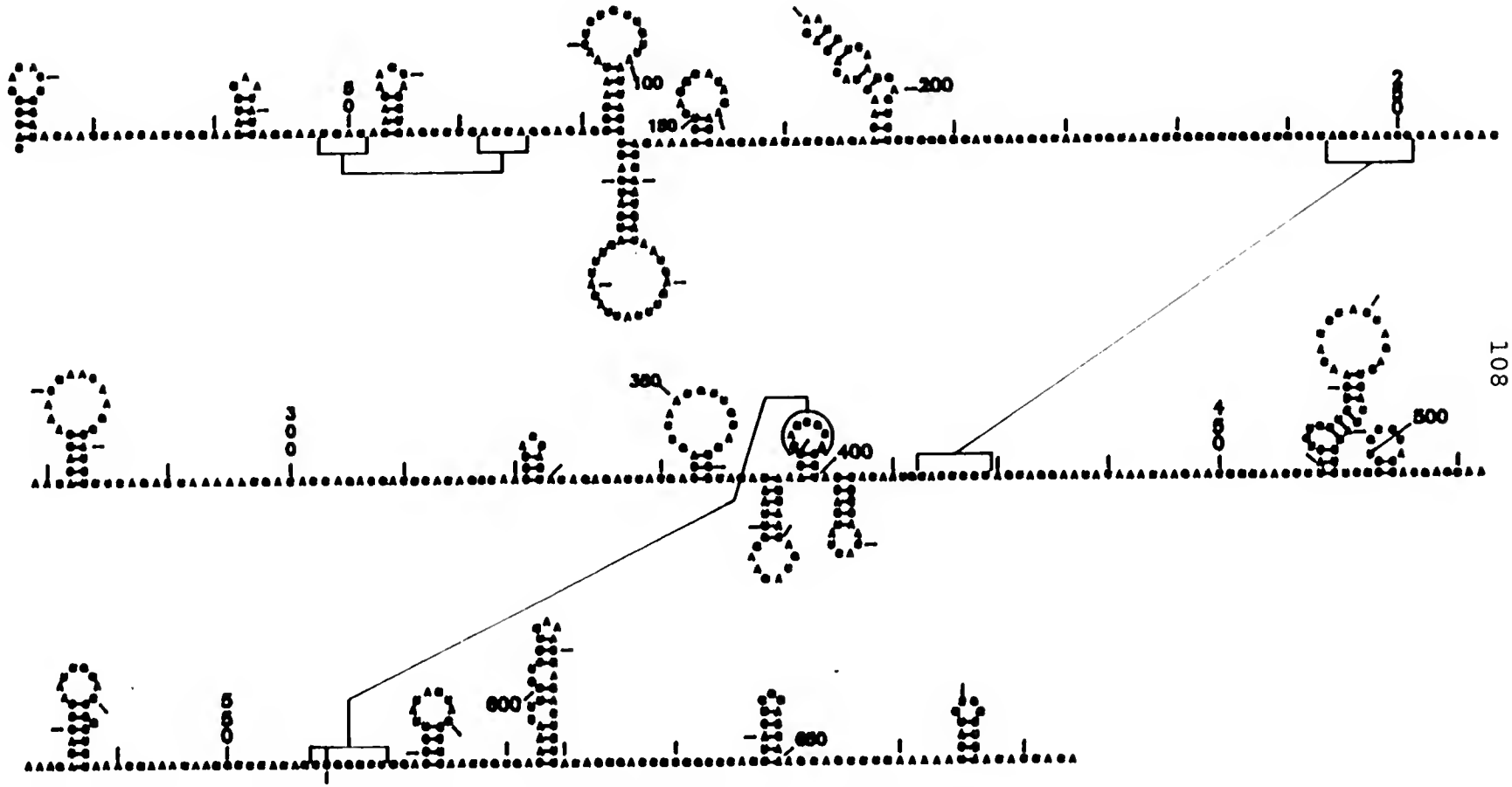


Figure 33. Potential Additional Structures on COII mRNA Model. Brackets indicate the extent of the base pairing and arrows connect the segments.

individual files (21 in all) and were used to search the entire COII sequence using the WORDSEARCH program. The results of the search produced a substantial amount of possible matches that were examined in reference to the predicted structure (Figure 32) and the modification data.

The possibility of three additional helical stems is suggested (Figure 33). These stems are simply indicated by brackets joining their sequences, because of the speculative nature of these interactions. Most interesting of this set of additional stems is the possible long-distance interaction between C245-C251 and G423-G428. This not only pairs unmodified portions of the molecule, but is also consistent with the V_1 modifications seen at 249 and 250. It would also have the effect of pulling the mRNA into a more condensed structure. A potential four base stem between bases U48-U51 and A72-A75 acts to extend an already predicted helical stem between G53-G56 and C62-C65. A pseudoknot type of interaction is suggested for the bases between C391-A397 and U560-U565. Here again the effect would be to draw the molecule into a more compact structure.

Conclusions

The best available method for disclosing RNA structure, modification/primer extension, and the best RNA folding program, LRNA, were used to predict the structure of COII

mRNA. Nevertheless, it must be remembered that these models remain predictions of the possible secondary structure of COII mRNA. If these additional base pairing interactions (Figure 33) occur, the COII mRNA would be folded into a "knot" which may sequester a significant portion of the molecule in stem structures or other domains. If the requirement for a 30 base binding site on the ribosome is to be met, then this folded mRNA may be displaying a limited number of targets for the ribosome. Long stretches (30 bases or greater) of single stranded nucleotides are only a few and some of these longer segments are found in large loops. It is not known whether it is possible for the small ribosomal subunit to bind these types of single stranded structures. The question of how or where the small subunit interacts with the message is not answered here, but a better understanding of what the 28S subunit "sees" as it approaches the message is provided as well as the method for locating its sites of interaction on the mRNA.

CHARACTERIZATION OF MITOCHONDRIAL RIBOSOMAL SMALL (28S) SUBUNIT INTERACTION ON THE COII MESSENGER RNA

Background and Significance of 28S Subunit Interaction with COII mRNA

A model for the COII message secondary structure, developed from both the physical probing by enzymes and chemicals and advanced computer predictions, provides a backdrop for the examination of mRNA-28S subunit points of interaction. "Points of interaction" is appropriate because individual bases are probed by this previously described and employed method. The structural model of COII serves the purpose of a visible representation of what the small subunit must associate with in order to initiate translation.

A number of investigators have looked at the secondary structural constraints of mRNA on initiation of translation in both prokaryotes and eukaryotes. The structure most commonly associated with eukaryotic ribosome-mRNA binding is the 5' cap, or methyl-G cap. This structure facilitates the binding of a protein, the cap binding protein, which in turn assists in the binding to the ribosome. However, not all mRNAs translated by eukaryotic ribosomes are capped, though they are translated. Picornavirus RNAs are not capped and use an

internal entry site for the ribosome with the aid of several proteins (eIF4A, eIF4B, p52 and p57)(Sonenberg, 1991). These messages may be aided in their initiation of protein synthesis by a "modest" amount of downstream structure (Kozak, 1990). This also has an advantage for messages with non-AUG initiation codons, such as GUG and UUG, and AUG codons that are located in unfavorable primary sequence contexts. In both of these cases the 40S subunit may bypass the "leaky" initiation codon and initiate at an AUG codon farther downstream. Mitochondrial mRNAs do not have 5' caps, nor do they have a primary sequence 5' of the initiation codon to provide a context, favorable or unfavorable. They bind equally well whether their 5' end nucleotide contains a triphosphate, monophosphate, or hydroxyl (Liao and Spremulli, 1990a). The effect of secondary structure in mRNAs on mitochondrial ribosome binding has not been examined.

It is a primary structure that is most frequently identified with prokaryotic message binding. The S-D sequence (4-9 base, purine-rich sequence located within 15 bases of the initiation codon) is a key factor in the binding of most prokaryotic mRNAs. This sequence has the important role of positioning the initiation codon near the peptidyl-tRNA binding site (Calogero et al., 1988). Messages that lack this sequence are still bound by 30S ribosomal subunits, albeit with a 10 fold loss in affinity (from $K_a = 2 \times 10^7\ M^{-1}$), can form initiation complexes, and are translated in vitro. The

S-D sequence appears to confer a functional advantage only at low concentrations of mRNA.

Other investigators showed that secondary structure around the initiation codon in prokaryotes is compensated energetically by a high affinity interaction between the message and the ribosome (de Smit and Van Duin, 1990). The interaction may act as a concentration effect for initiation codons near the peptidyl-tRNA binding site. However, mitochondrial mRNAs do not have S-D-like 5' leader sequences, nor does the 12S rRNA have an anti-S-D sequence at its 3' terminus, suggesting that they utilize a different mechanism for binding to ribosomes.

Mitochondrial mRNAs are bound by 28S ribosomal subunits in the presence and absence of initiation factors. They are translated *in vivo* without the aforementioned advantageous structures used by prokaryotes and eukaryotes. The small subunit has been shown to have an mRNA binding domain that accommodates thirty bases and binds polyribonucleotides with high affinity (Denslow *et al.*, 1989).

In this work we have shown that 28S subunits bind all tested mRNAs equally well (K_d about 5×10^{-8}M), with the exception of poly(C) (2- to 3-fold weaker), and apparently they all bind to the same site on the ribosome. Also, similar to all other ribosomes, 28S subunits appear to require single stranded regions of RNA for binding. Only one ribosome appears to bind to a single mRNA under the conditions used.

The binding is reversible and it is inhibited by a known inhibitor of proteinaceous RNA binding sites. A significant amount of the mRNA seems to be involved in base pairing/secondary structure, leaving only a few segments of thirty base or longer as candidates for primary binding sites.

The tools used to elucidate the secondary structure are the same as those needed here to examine the ribosome-mRNA site of interaction. The modification/primer extension method will be combined with the conditions discerned for binding interactions between COII mRNA and 28S subunits. The binding stoichiometry leads to the expectation that a single site of ribosome interaction exists on the message.

Footprints of 28S Subunits on COII mRNA

To study the binding site for ribosomes on the COII mRNA, conditions were chosen to ensure that only one subunit would be bound to the mRNA (see Figure 12). The condition used, 9 pmoles of COII mRNA and 18 pmoles of small subunits, is very near the saturation plateau (75-80% of saturation) and ensures the stoichiometry and the economy of the reactions. These are incubated for 5 minutes at 35°C to allow for binding and for an additional 10 minutes after the introduction of a chemical or enzymatic modifier.

The effect should be to modify nucleotides differentially depending on whether or not small subunits are associated with

the message. Bases on the mRNA that are in contact or proximal to the interaction between the ribosome and message should be less accessible to modification than those outside the region or than those modified in the absence of small subunits. Sites of these differential modifications were then disclosed by primer extensions with AMV-RT.

The modifiers expected to be most sensitive to small subunit interaction were the nucleases, A and T_1. The size of these modifiers, along with the recognition requirements of the nucleases, should keep them from affecting the mRNA at the bases which are in intimate contact with the 28S subunit. The nucleases must not only recognize the base but interact with the phosphate backbone in order to cleave the phosphodiester bond.

An additional effect frequently seen when this technique is used to disclose RNA binding sites is the presence of enhanced bands in the presence of the binding entity. This generally occurs on the periphery of the "binder" (i.e., the ribosome) at the site of interaction and, thereby, bracket the site.

The modification by the very reactive DMS may not be so easily arrested by the interaction of the ribosome on the mRNA. The very small size of the molecule allows it freer access to all bases exposed to solution. Furthermore, it reacts not so much with a base as with a portion of that base (see Figure 4). Thus, the base may be in a region where

interaction with the ribosome is occurring and yet remain quite accessible to modification by DMS.

It was expected from the earlier binding stoichiometry data that there would be a single site of interaction. Expectations from the previously described work (Denslow et al., 1989) were that the interaction might be at the 5' end. This does not preclude other areas being in contact, nor does it preclude having some domains that are not in contact but are sequestered by ribosome interaction. Both of these would reflect a reduction in modification and might be predicted of a "knotted" message.

As can be seen in Figures 34-38 many bases are differentially modified under these conditions. RNase A was seen to have diminished effects in a clustered site, while RNase T_1 signals were affected over a more widely scattered array of guanines.

In order to quantify the effect of 28S subunits on the modifications, each of the affected bands in the autoradiography was scanned using the General Imaging Scanner of the Protein Core Facility (ICBR). An example of the densitometry is shown in Figure 39; results of all scans are displayed in tabular form in Table 10. The percent of modification reduced is the result of subtracting the control value for the particular band from the diminished (plus 28S subunits) modified band of interest and then dividing by the similarly adjusted undiminished (minus 28S subunits) modified

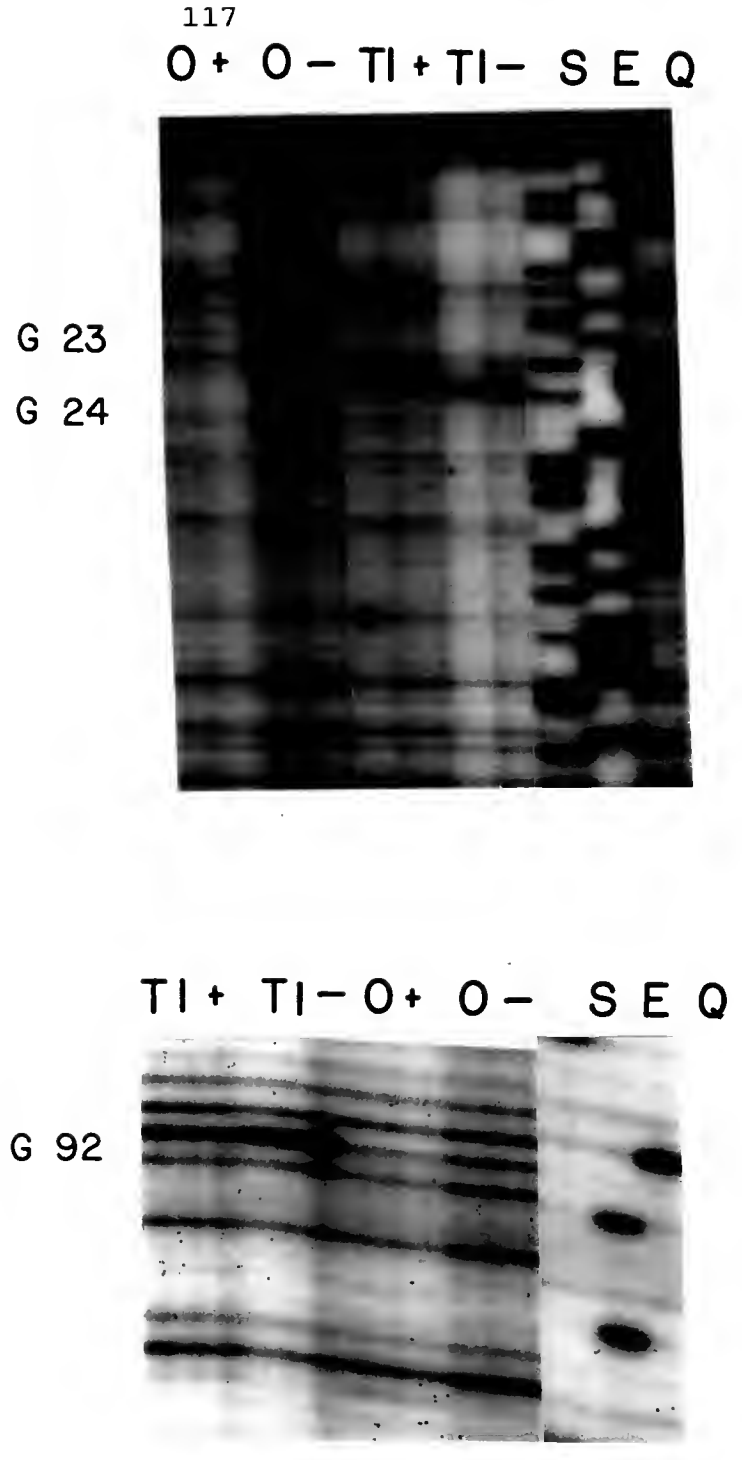


Figure 34. Reduced Modifications of RNase T_1 on Ω Extensions. 0: control- no nuclease; T_1: RNase T_1 modified; +: presence of 18 pmoles of 28S subunits; -: no ribosomes; and SEQ: A,C,G sequencing ladder.

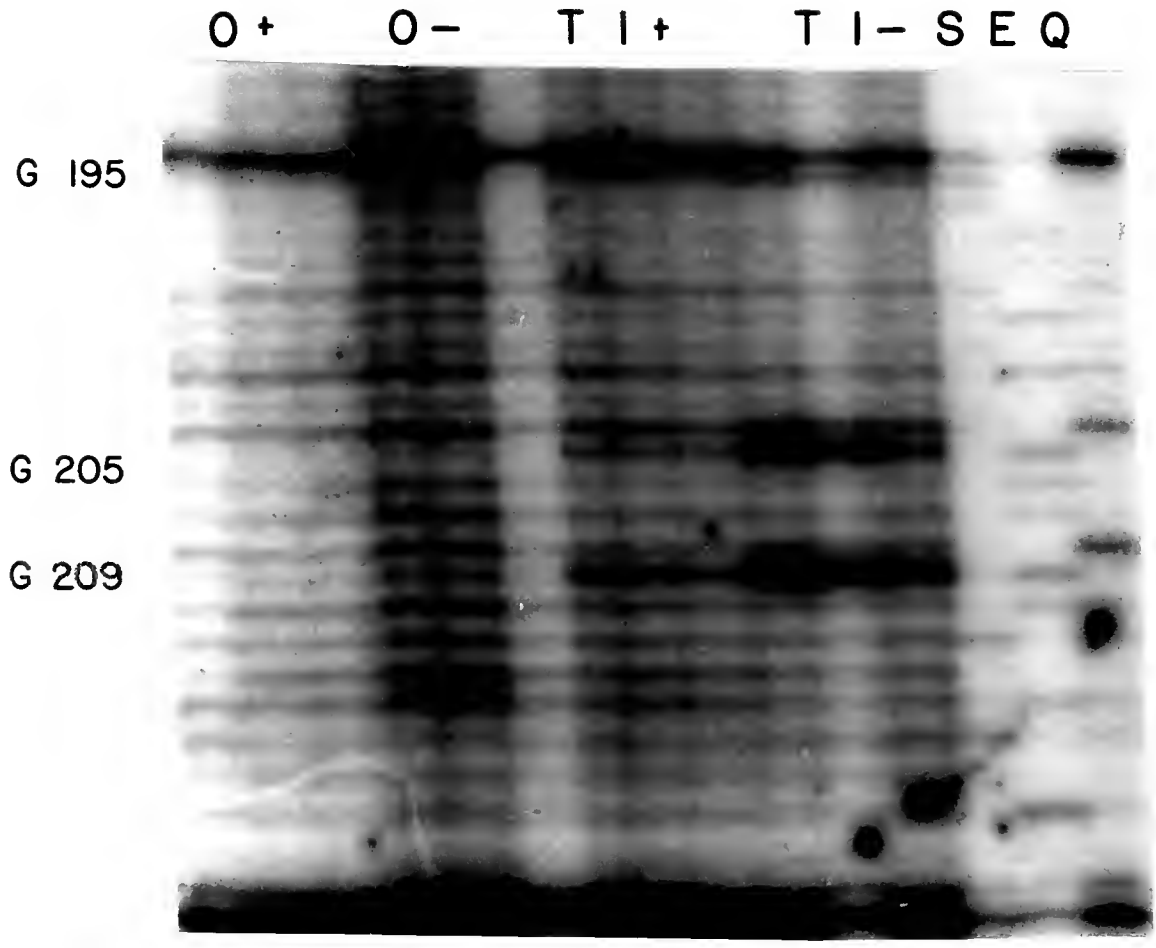


Figure 35. Reduced Modifications of RNase T_1 on α Extensions. 0: control- no nuclease; T_1: RNase T_1 modified; +: presence of 18 pmoles of 28S subunits; -: no ribosomes; and SEQ: A,C,G sequencing ladder.

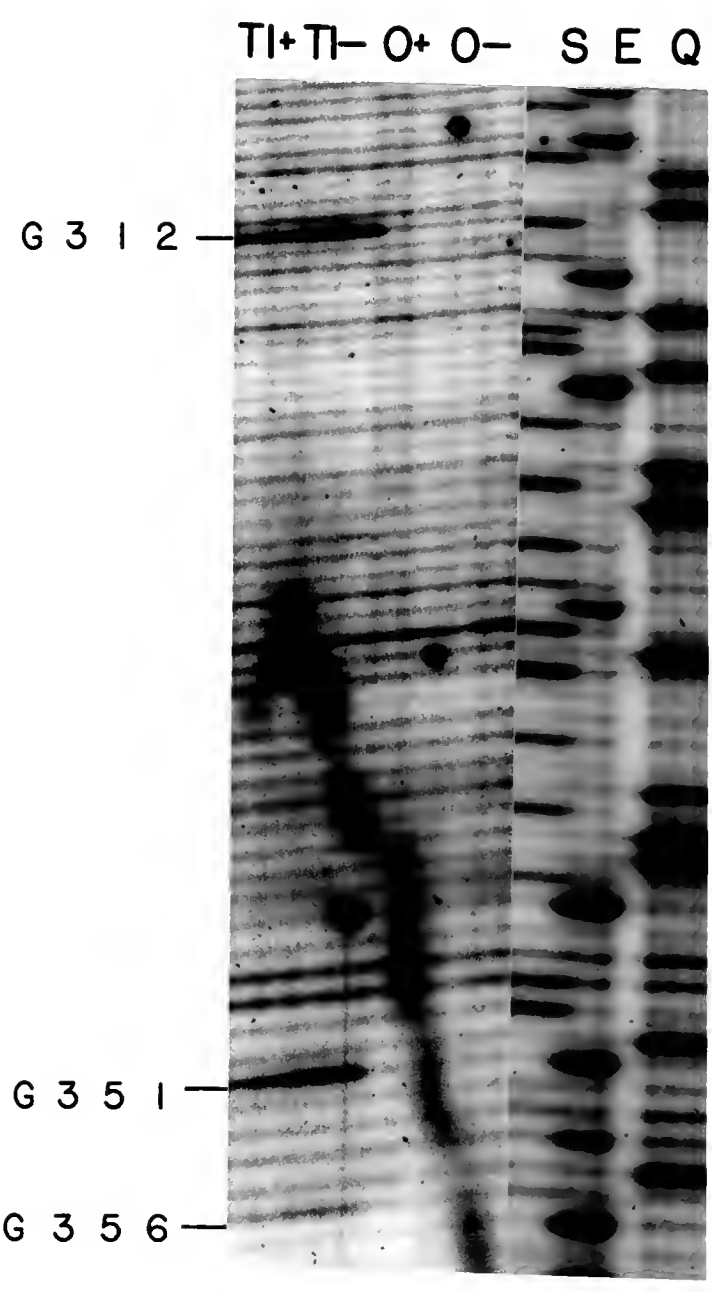


Figure 36. Reduced Modifications of RNase T_1 on β Extensions. 0: control- no nuclease; T_1: RNase T_1 modified; +: presence of 18 pmoles of 28S subunits; -: no ribosomes; and SEQ: A,C,G sequencing ladder.

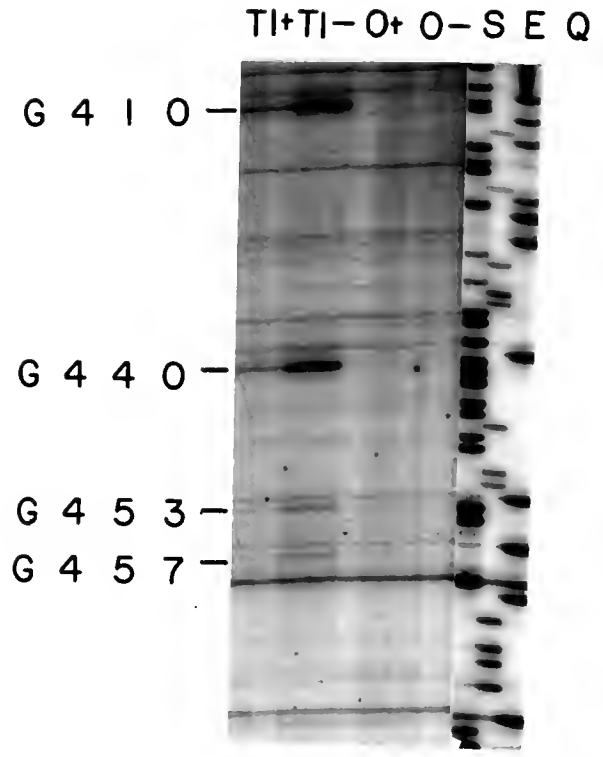


Figure 37. Reduced Modifications of RNase T_1 on γ Extensions. 0: control- no nuclease; T_1: RNase T_1 modified; +: presence of 18 pmoles of 28S subunits; -: no ribosomes; and SEQ: A,C,G sequencing ladder.

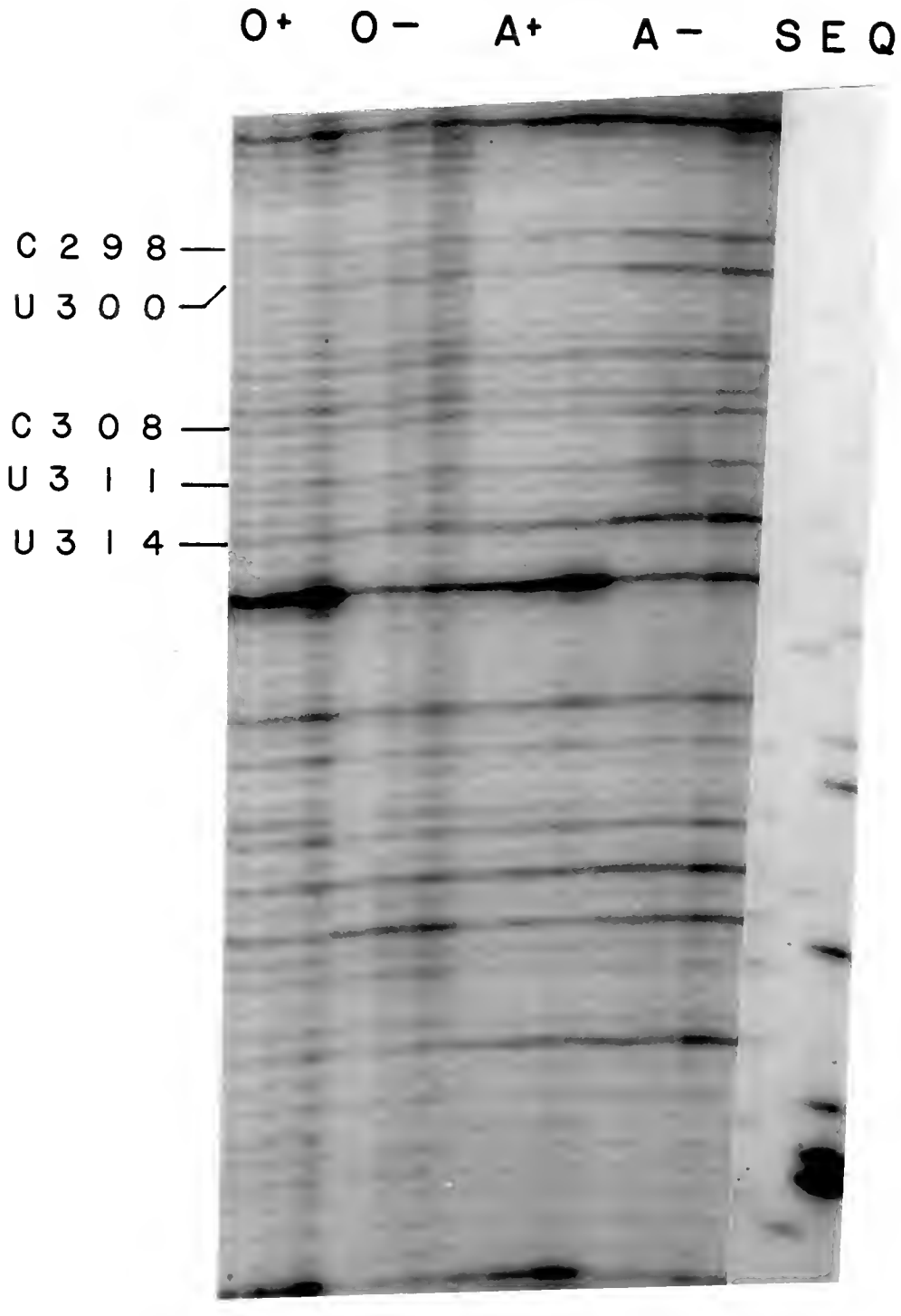


Figure 38. Reduced Modifications of RNase A on β Extensions. 0: control- no nuclease; A: RNase A modified; +: presence of 18 pmoles of 28S subunits; -: no ribosomes; and SEQ: A,C,G sequencing ladder.

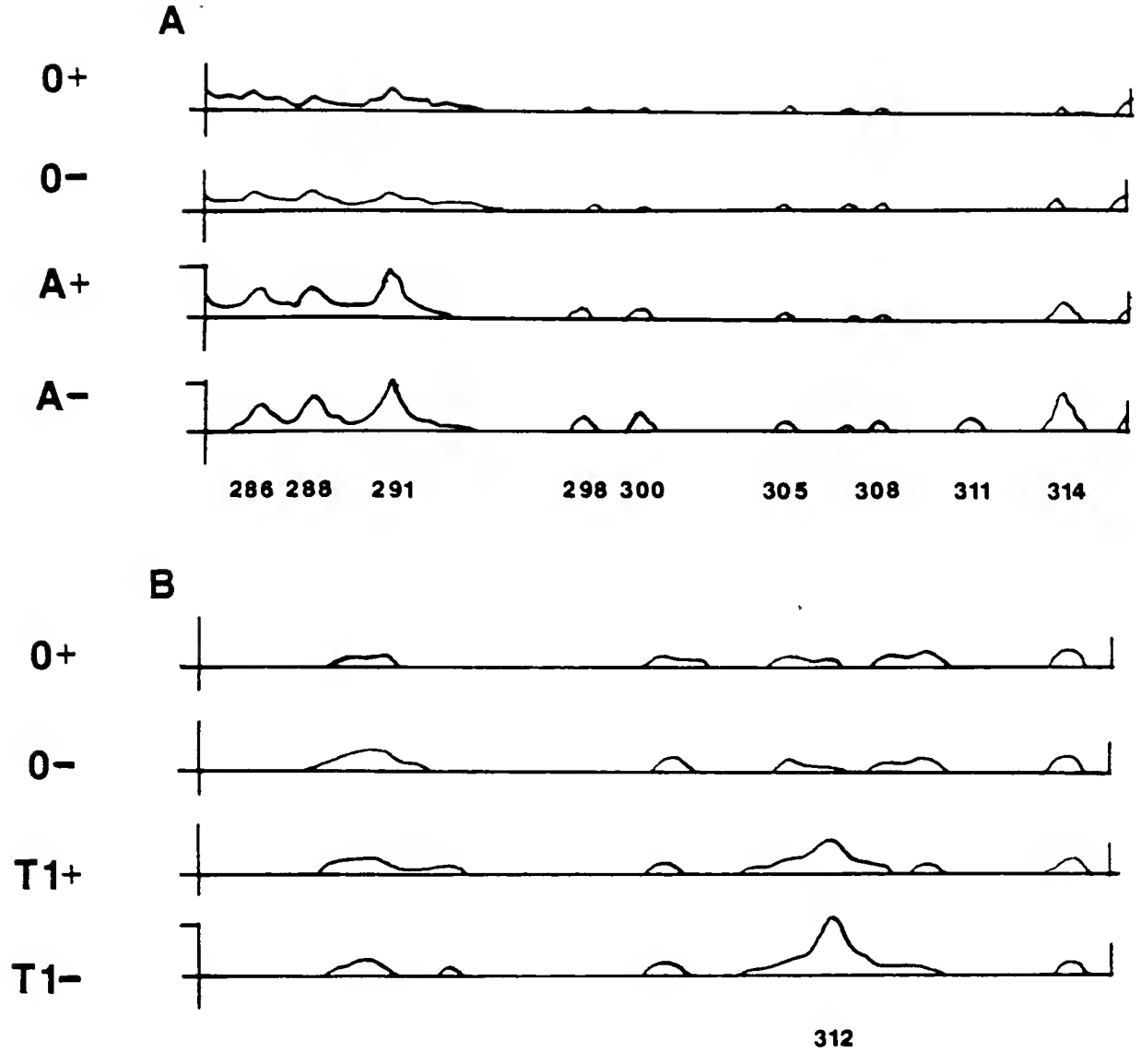


Figure 39. Densitometry of Modification Reductions of RNase A and T_1 on β Extensions.

Table 10

Effects of Binding to 28S Subunits on Enzymatic Modifications to COII mRNA.
Analysis of the Scanning Densitometry of Autoradiographic Bands of Diminutions.

Modifier	RNase T_1		RNase A		Control		Percent Reduction
+/- 28S	+	-	+	-	+	-	
Base/#	--------		--------		--------		--------
G 23	0.26	0.51	--------		0.06	0.06	55.6
G 24	0.26	0.70	--------		0.05	0.03	68.7
G 92	0.50	0.98	--------		0.23	0.26	62.5
G 195	0.19	0.31	--------		0.05	0.05	48.2
G 205	0.26	0.86	--------		0.00	0.00	69.8
G 209	0.44	1.16	--------		0.00	0.00	62.1
C 288	--------		0.19	0.31	0.07	0.07	50.0
C 298	--------		0.05	0.09	0.02	0.02	57.1
U 300	--------		0.06	0.13	0.02	0.03	60.0
C 308	--------		0.03	0.04	0.04	0.12	87.5
U 311	--------		0.02	0.02	0.05	0.13	72.7
G 312	0.14	0.39	--------		0.07	0.16	69.6
U 314	--------		0.16	0.32	0.04	0.05	55.6
G 351	0.12	0.38	--------		0.09	0.09	88.9
G 410	0.40	1.01	--------		0.10	0.10	67.0
G 440	0.28	0.80	--------		0.05	0.05	69.3
G 453	0.05	0.16	--------		0.05	0.05	>99.9
G 457	0.04	0.12	--------		0.04	0.04	>99.9

band. All but two, G195 and C288, are between 55-75% diminution and correlate fairly well with the 75-80% binding expected under these conditions. This is true, especially when considering the dynamic nature of binding interactions over a 15 minute period. One might expect that the mRNA would move on and off the 28S subunit several times in this period making a base protected from modification by the small subunit susceptible to being modified. The guanines at 453 and 457 were initially weak modifications that were totally abrogated. The complete protection from modification may simply be a effect of their original near insusceptibility.

Characterization of the 28S Subunit Interaction Site on COII mRNA

For ease of viewing and further analysis the sites of diminished cleavages were plotted on the COII mRNA secondary structure model (Figure 40). One may observe that RNase T_1 was more readily hindered in its modifications than RNase A and DMS reactive sites do not appear to be affected by the presence of the ribosome. The size difference is negligible between RNase A and T_1 (13.7 and 11.0 kd, respectively), but perhaps their requirements for recognition and modification are different enough to render RNase T_1 more sensitive to the presence of another molecule.

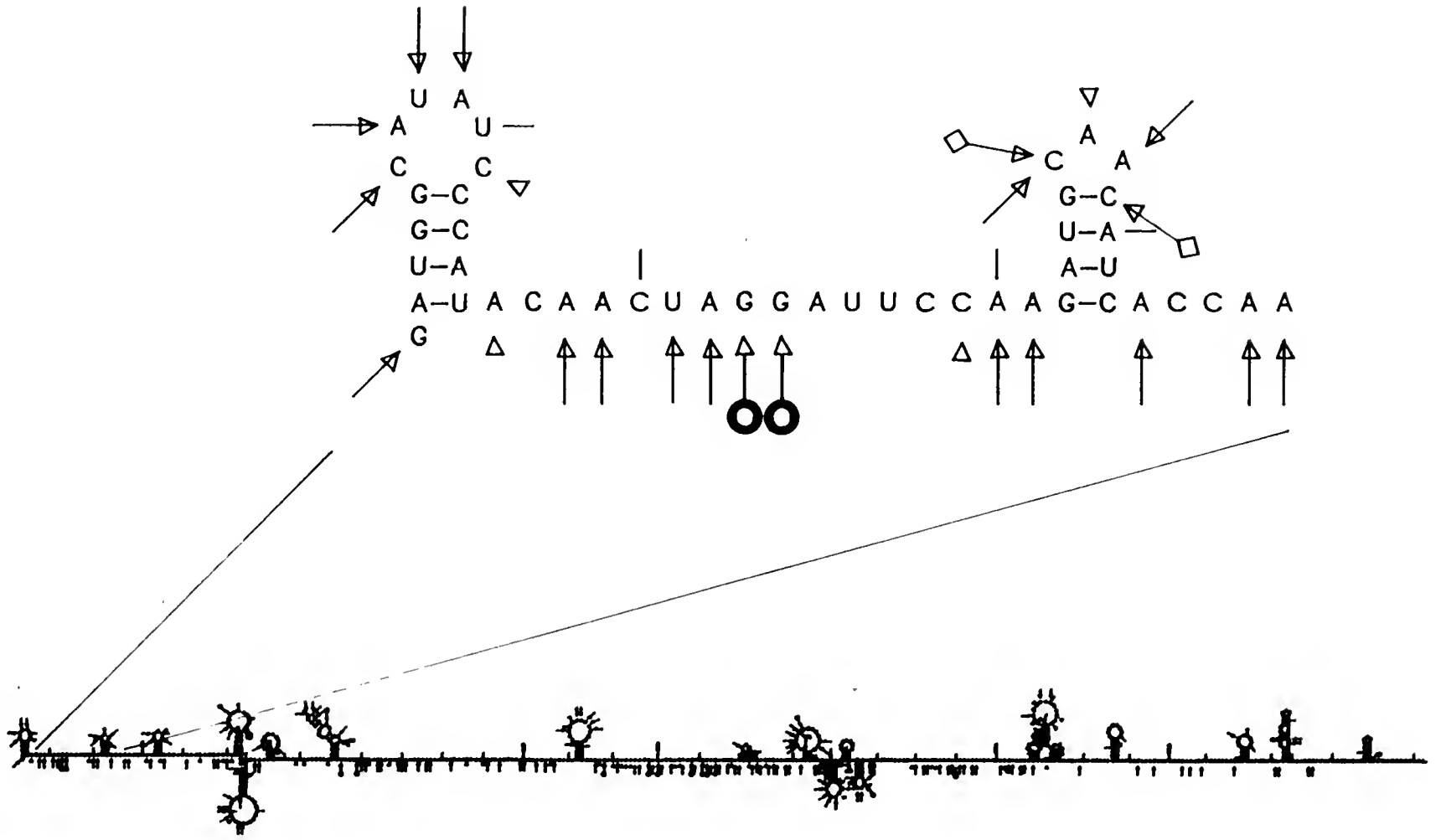


Figure 40. Diminished Modifications on the CoII mRNA Secondary Structure Model. Circles indicate the modifications that were reduced when 9 pmoles (0.18μM) of CoII mRNA were incubated in the presence of 18 pmoles (0.36μM) of 28S subunits for 5 minutes at 35°C and then incubated an additional 10 minutes at 35°C in a final volume of 50μl. Diamonds indicate enhanced modification in the presence of ribosomes.

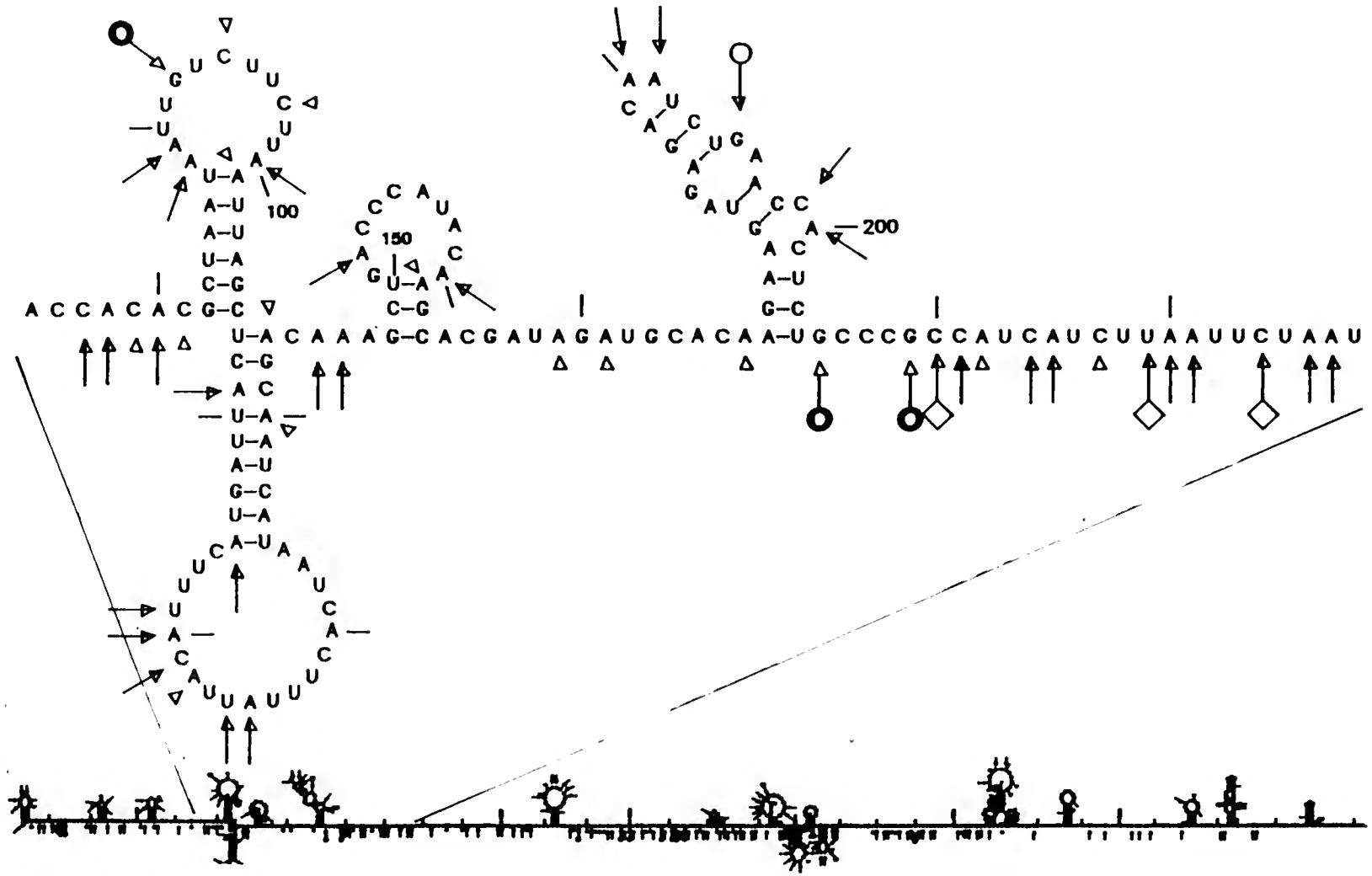


Figure 40. (cont)

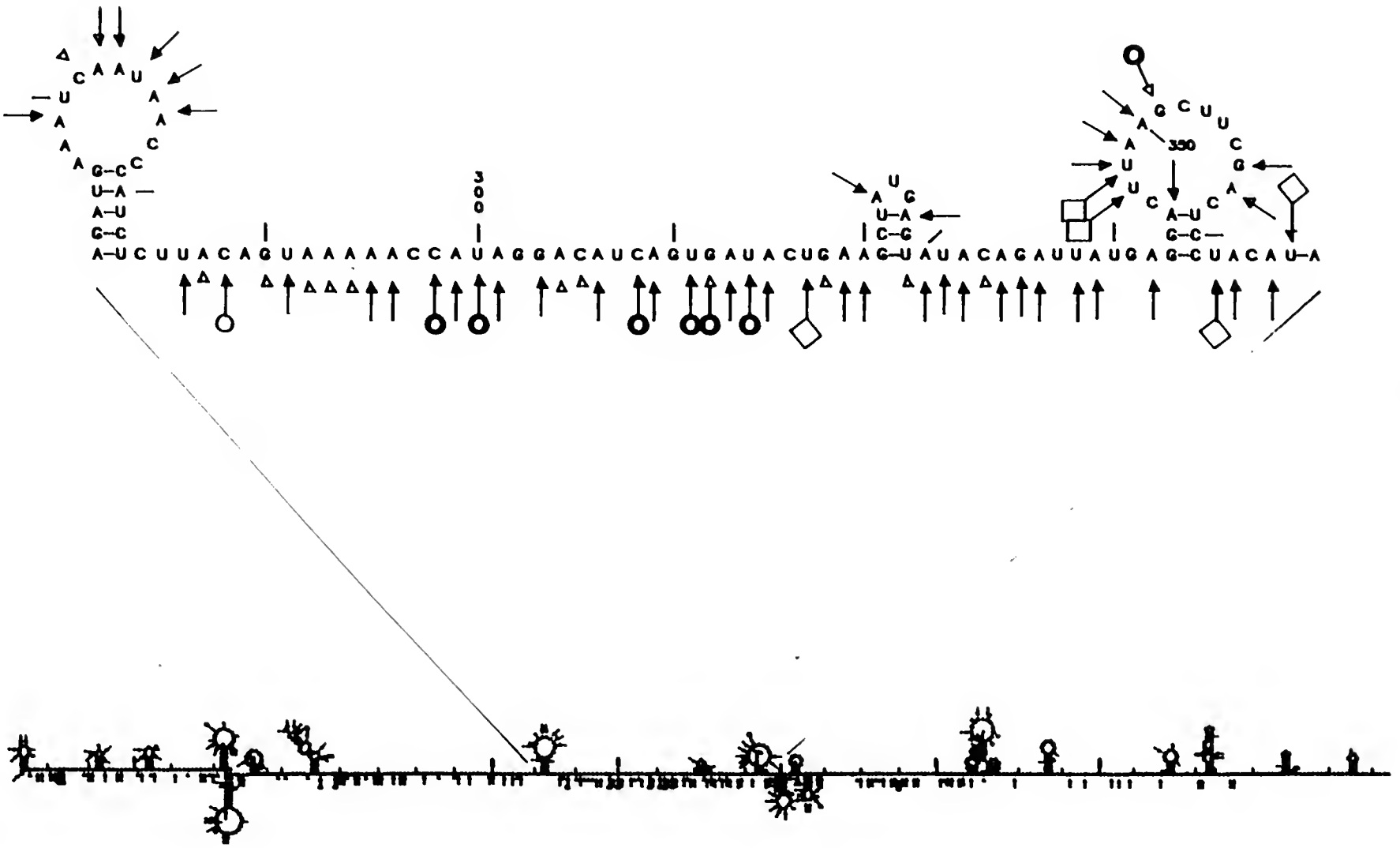


Figure 40. (cont)

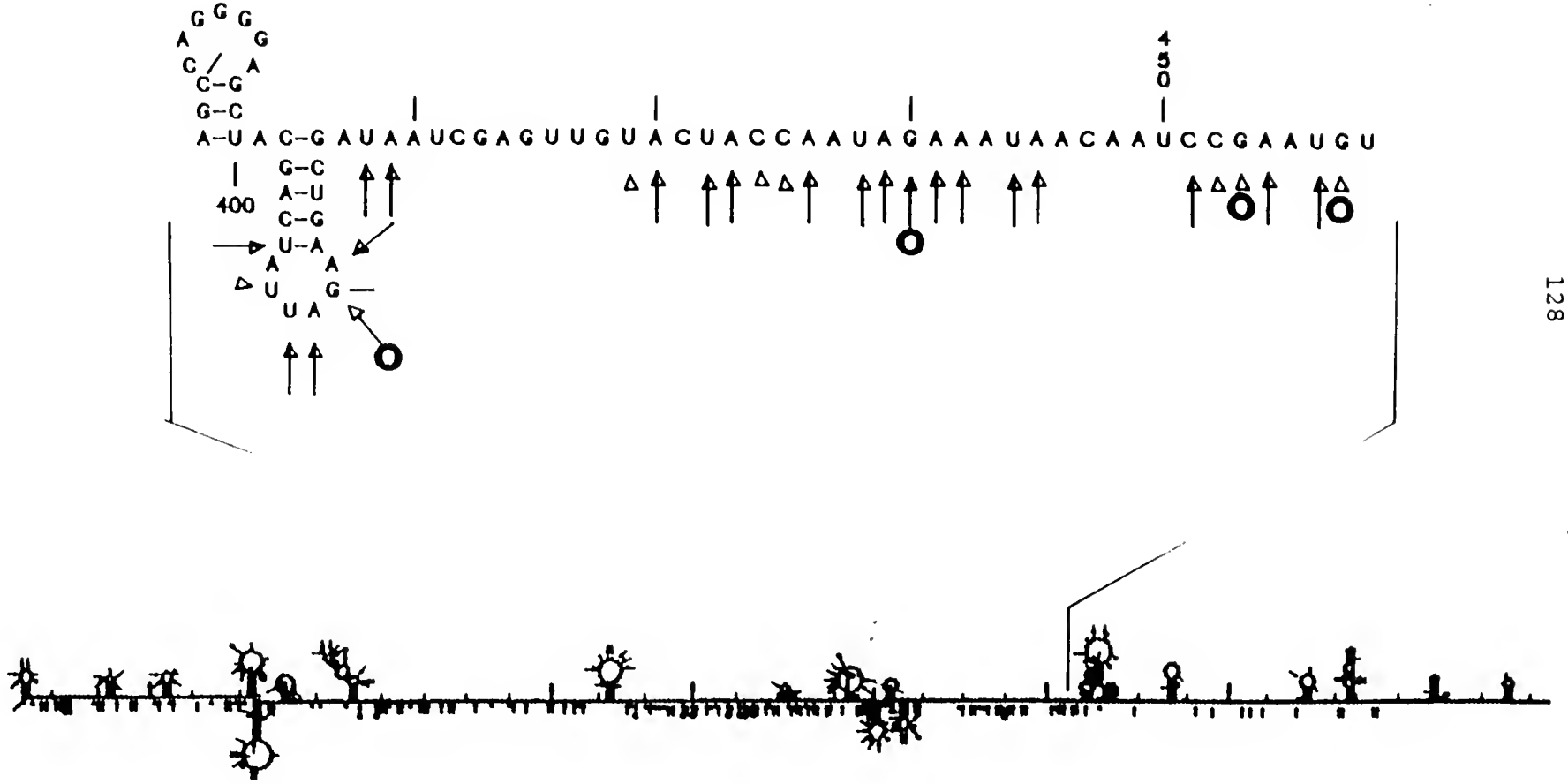


Figure 40. (cont)

It is the long stretches of single stranded RNA that are most frequently reduced in their accessibility to nuclease modifications. It is clearly seen that the 37 base stretch between U283 and U320 shows diminished modifications of seven out of thirteen nuclease susceptible bases. These cleavages show a periodicity of 8-11 bases which may indicate a helix like the A-type helices common to RNAs (Saenger,1984). For this to occur only one side of the helix would actually be interacting with the small subunit in this region, though this need not be the case.

Some of the other diminished modified nucleotides (G94, G351 and G410) have the conundrum of being in loop structures. There is no evidence of small subunits binding circular or loop constrained RNA and they may not actually be in contact with the ribosome here. If the interaction predicted for a stem structure between C245-C251 and G423-G428 did indeed occur, then the RNase T_1 modifications and subsequent diminutions at guanines 195, 205, 209 and 410, 440, 453, 457 would be in proximity one with another. The protection domain, much larger than the 80 bases previously described, may be the result of regions like this that are sequestered in RNase T_1 inaccessible pockets. Another possibility that has been proposed (Liao and Spremulli, 1990b) to explain the apparent inability of the message to be bound by more than one 28S subunit is that the message wraps around the ribosome.

It should be noted here that though only G23 and 24 were protected at the 5' most end of the molecule this result does agree with the results previously described of 5' end protection of mRNAs by the 28S subunit (Denslow et al., 1989).

Furthermore, the reduction of nuclease activity could possibly be the result of a shift in the secondary structure of the mRNA molecule induced by the interaction with the ribosome. All of the modifiers used were structure dependent so that binding or a change in structure might look the same. This may be the case but one would expect a new set of modifiable or cleavable bases to be disclosed. Also, when the binding of a ribosome involves the "removal" of structure (picornavirus mRNAs)(Sonenberg,1991) additional protein factors are required.

Conclusions

The results show that the effect of one 28S subunit binding to COII mRNA at near saturating amounts displayed an ability to reduce modifications almost stoichiometrically to several bases in the 5' two-thirds of the message. In accordance with the binding assays done in Chapter I and data compiled in both eukaryotic and prokaryotic systems, it appears that mitochondrial small subunits bind to single stranded stretches of RNA. A region between U283 and U317

showed the most extensive number of modification reductions over a 30+ base stretch of apparent single stranded RNA.

Work had been done on the binding of shortened COII mRNA that showed that 5' end fragments 250 bases in length or shorter were able to bind less than 50% of maximal under the conditions used (similar to those used here) (Liao and Spremulli, 1990c). The addition of the next 103 bases, out to U353 conferred near maximum binding. No additional benefit was apparent above 500 bases. This seems to indicate that 350+ bases are required of COII mRNA for competent binding by the small subunit and may at first glance appear to contradict the optimum binding length of 30 bases and protection of up to 80 bases established earlier by binding studies with oligoribonucleotides (Denslow et al., 1989).

The model proposed here is that the 28S subunit contains a binding site capable of accommodating 30 bases and protecting considerably more. The length effect observed in the shortened message experiments is probably the result of the structured nature of the 5' end of COII mRNA. The site of interaction with the small subunit involves the 5' two-thirds of the molecule and particularly the stretch of bases between U283 and U317. Furthermore, protection is conferred to additional areas of the mRNA not necessarily by direct contact but by limiting the access of modifiers by either domain sequestration or perhaps a wrapping of the message around the 28S subunit.

It should be noted that the 5' end of the molecule is only modestly protected in these studies. The method used could not determine if the structure predicted for 5' fifteen bases is protected or simply remains in the same stem-loop structure. How the 28S subunit locates the initiation codon remains a mystery and may require the addition of as yet isolated and characterized "factors."

CONCLUSIONS AND FURTHER DIRECTIONS

Summary

Binding studies between 28S subunits and various nucleic acids provided information regarding the specificity, stoichiometry, and affinity of the small subunit for these molecules. The ability of the ribosome to bind the messages tested with an apparent affinity of 5×10^{-8}M and unit stoichiometry showed that there was probably a single site for ribosome-RNA interaction on the 28S subunit, as well as, an apparent single site on the message. The binding was reversible, saturable, and all messages tested competed for the same site on the small subunit. Since the binding of poly(U) by 28S subunits has been shown to be functional *in vitro* (Denslow and O'Brien, 1979; Kumazawa *et al.*, 1991) and this homopolymer competes effectively with COII mRNA for binding on the same subunit, the expectation is that this binding site is functional as well.

The apparent binding affinity for nucleic acids other than messages was significantly reduced, greater than 20 fold. This indicated that double stranded polynucleotides do not interact significantly with the small subunit and that the nature of single stranded DNA was different enough to impede

substantial binding. Inhibition of the binding of COII mRNA by the 28S subunit is caused by aurintricarboxylic acid, an inhibitor of RNA binding proteins, leads to speculation into the potential proteinaceous nature of the mRNA binding site on the 28S subunit. The apparent lack of a S-D-like sequence on the COII mRNA did not affect its ability to be bound by the small subunit, which also lends credence to the idea that the site may be proteinaceous. Otherwise, an unusual and yet to be described type of interaction must be occurring, but this, too, is possible, considering the many atypical features of the mitochondrial translation system.

Chemical and enzymatic probing and computer modelling allowed speculation into the secondary structure of COII mRNA. In this manner the "terrain" upon which the 28S subunit must bind was mapped and then folded into "best" prediction by the latest computer folding and comparison algorithms. This model discloses a predicted structure for COII mRNA that possesses a limited number of single stranded stretches that might correlate to the proposed 30 base binding site (Denslow, et al., 1989).

Chemical and enzymatic probing was employed under conditions that support the interaction between the COII mRNA and the 28S subunit. The probing determined the nucleotides that are interacting with or obscured by the 28S subunits binding to the message. On this basis, a putative binding site on the mRNA of COII was located between U283 and U317

consistent with the data purporting the 30 base binding site and the requirement for the 5' two-thirds of the molecule for significant binding of the message. The reductions of nuclease effects show a periodicity of 8-11 nucleotides or about what would be expected of an A-type RNA helix. Perhaps the message is coiled but otherwise unstructured in this area providing a single stranded stretch which is protected from nuclease cleavage on only one side of the coil.

Future Directions

Like most studies, this project raises a number of questions that might be further explored. The first section, which considered the nucleic acid binding properties of the 28S subunit, could be expanded to include other mRNAs such as capped and polyadenylated eukaryotic mRNAs to determine what constraints these features may impose on the binding. The probing of other mitochondrial messages bound to 28S subunits would also narrow some of the particular requirements of the mRNA for ribosome binding .

The message for COII could undergo deletion analysis of the various individual regions of modification to determine the effects of each on the binding by 28S subunits. This would provide information about the affinity of the small subunit in the absence of all or some of the regions of reduced modification.

If mammalian mitochondrial regulatory factors exist and previous work suggests they might (Denslow et al., 1989), they may be similar to PET 122 and CBS 1 discovered in yeast (Saccharomyces cerevisae), which bind a specific mRNA and present it to the small subunit for the initiation translation (McMullin et al., 1990; Costanzo and Fox, 1990). Additionally, an S1 (prokaryote) like protein may be present in the mammalian mitochondria to facilitate the initiation of translation. The mitochondrial messages could be used for isolating analogous factors from mammalian mitochondria by affinity chromatography or in assays for binding. The binding and modification techniques could be used in the presence of isolated mammalian mitochondrial regulatory factors to determine the strength and site of interaction on the message. Initiation factors for the mitochondrial translation system continue to be of interest since to this point only one (IF2) has been isolated (Liao and Spremulli, 1990a). The search for mammalian mitochondrial factors and their sites of interaction is the particular scope of the investigations of Scott Fiesler in our lab.

Directionality of the introduction of the message into the binding site on the 28S subunit as well as the characterization of the participants are also of extreme interest for this system. This can be attained by cross-linking (UV) a labelled mRNA to the ribosomal subunit and is the ongoing work of Robert Heck in the lab. The binding and

cross-linking has disclosed two individual components (proteins) of the ribosome. It may be possible to label the mRNA in a manner that would be more readily visible by electron microscopy using biotinylatable nucleotide analogs. This would allow a general localization of the binding site on the small subunit.

A three dimensional model of the COII message may provide some insight into the way that domains are presented to the 28S subunit. A model of both the message and analogues provide clues to the way regions of the message are sequestered in the presence and absence of the ribosome. A model of this type for the 28S subunit is in process and is the effort of Dr. Wes Faunce in our lab.

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BIOGRAPHICAL SKETCH

Bernard Clark Courtney was born on 18 November 1957, in Great Lakes, Illinois, the son of Bernard and Wilma Courtney. He was raised in a military family, changing schools seventeen times before graduating from high school. He graduated from Auburn University in 1980 with a B.S. in Agriculture (Agronomy). He also attended Trinity Evangelical Divinity School. In the tradition of his father he joined the Navy being commissioned as an ensign through Aviation Officer Candidate School in July, 1983. He has maintained this affiliation as an active drilling reservist for the Atlantic Intelligence Command 0774 at NAS Jacksonville and was recently selected for Lieutenant Commander. He was married to Janet Marie Tobin in November, 1984 and is now the proud father of three boys--Ryan Clark (5), Sean Michael (3), and Quinn Stephen (1). His immediate plans include transferring his commission to the Army where he will conduct research on immunologic and biochemical countermeasures to chemical and biological weapons at the US Army Medical Research Institute for Chemical Defense in Aberdeen, Maryland.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1992

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